REMARKS

The present invention stems from Applicants' pioneering discovery that fully assembled antigen-specific immunoglobulin can be produced in a plant cell. The inventors also were the first to achieve a level of expression that makes possible passive immunization with plant produced antibodies. Plant produced antibodies are useful for systemic protection through administration i.v. as well as localized protection through local administration to a mucosal surface (e.g., lungs, digestive tract, nasopharyngeal cavity, the urogenital system).

The claims are generally directed to methods of passive immunization by administering an immunoglobulin produced by transgenic plant cells. Claims 13, 15-27 and 21-91 are pending.

Applicants have amended claims 13, 41 and 83 to add emphasis in the preamble of the claim that the method of passive immunization involves administering an immunoglobulin produced in plant cells. No new matter is introduced by the amendment as it merely reflects steps in the body of the claim. Claim 83 has also been amended by deleting reference to the invention deemed withdrawn by the examiner. This amendment also does not raise issues of new matter.

REJECTION UNDER 35 U.S.C. § 112

The rejection of claims 29-30, 48-50 and 86-88 under 35 U.S.C. § 112, first paragraph, as containing subject matter not described in the specification in a manner that conveys to one skilled in the art that the inventors had possession of the claimed invention is respectively traversed. It is alleged that the phrase "at least a portion of a heavy chain constant region" lacks written description in the specification

To satisfy the written description requirement of 35 U.S.C. § 112, ¶ 1, the specification must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, the applicant was in possession of the claimed invention. *See, e.g.*,

Vas-Cath, Inc. v. Mahurkar, 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d 1111, 1117 (Fed. Cir. 1991). Compliance with the written description requirement is essentially a fact-based inquiry that will necessarily vary depending on the nature of the invention claimed. *See, e.g., Enzo Biochem. Inc. v. Gen-Probe Inc.*, 296 F.3d 1316, 1324, 63 U.S.P.Q.2d 1609, 1612 (Fed. Cir. 2002).

Before addressing the teachings of the specification, Applicants wish to clarify an apparent misunderstanding held by the examiner, with respect to the view that "'at least a portion" of the constant region encompasses polypeptides having a few and one amino acid. This is incorrect. The claims require an "antigen-specific immunoglobulin molecule" or immunologically active fragment. This means that the heavy chain must have a complete or nearly complete variable region, which typically constitutes about 100 or so amino acids. Thus, even if the limitation "at least a portion of a heavy chain constant region" were to read on a single amino acid from the heavy chain constant region, such heavy chain polypeptide would be able to form an active site with light chain and would constitute a polypeptide of about 100 amino acids or so in length – not a single amino acid as asserted by the examiner.

Turning now to an analysis of the specification, the support for the phrase "at least a portion of a heavy chain constant region is found, for example, at page 10, line 27-33 (emphasis added).

> Immunoglobulin product: **A polypeptide, protein or multimeric protein containing at least the immunologically active portion of an immunoglobulin heavy chain and is thus capable of specifically combining with an antigen**. Exemplary immunoglobulin products are an immunoglobulin heavy chain, immunoglobulin molecules, substantially intact immunoglobulin molecules, **any portion of an immunoglobulin** that contains the paratope, including those portions known in the art as Fab fragments, Fab' fragment, $F(ab')_2$ fragment and Fv fragment.

Reference in the above to "at least the immunologically active portion of an immunoglobulin heavy chain" is an implicit reference to heavy chain variable regions that have any number of constant region residues. This language demonstrates that any and

all fragments were contemplated, limited only by the requirement that the fragment be "immunologically active." The exemplied (i.e. non limiting) examples include Fab' and Fab'2, which have CH1 domain and part of the hinge region or basically about 1 third of the constant region heavy chain amino acids, while the Fv fragment has a heavy variable region and no constant region amino acids. The above clearly contemplates immunoglobulins with heavy chains that have any number of constant region amino acids from zero to about 1/3 of the constant region residues in the exemplied non-limiting embodiments.

This view is additionally supported by page 3, lines 1-6 (emphasis added) of the specification.

> One of the most useful aspects of using a recombinant expression system for antibody production is the ease with which the antibody can be tailored by molecular engineering. **This allows the production of antibody fragments and single-chain molecules, as well as the manipulation of full-length antibodies**. For example, a side [sic] range of functional recombinant-antibody fragments, such as Fab, F_v, single-chain and single-domain antibodies, may be generated.

This passage indicates that recombinant expression makes possible the production of a variety of antigen-specific immunoglobulins including those known from proteolytic processing (e.g., Fab) and those known only by recombinant expression of light and heavy chain variable regions (e.g., single chain antibodies). The word "including" indicates that other molecules with portions of a heavy chain constant region were contemplated.

Furthermore, the ordinary skilled artisan would have appreciated that recombinant DNA methods as described in the instant patent specification can be used to produce immunoglobulins with heavy chains that include at least a portion of the heavy chain constant region. This is evidenced by the state of the art as of the earliest filing date of the instant application. For example, U.S. Patent no. 4,816,567 to Cabilly et al., filed April 8, 1983, describes the use of recombinant DNA technology to express antibodies that have less than a full length heavy or light chain (Summary of the Invention; emphasis added).

> The invention relates to antibodies and to non-specific immunoglobulins (NSIs) formed by recombinant techniques using suitable host cell cultures. . . .
>
> Finally, either the light chain or heavy chain alone, **or portions thereof**, produced by recombinant techniques are included in the invention and may be mammalian or chimeric.

Cabilly also teaches recombinant expression of any and all immunologically active fragments by referring to expressing "at least the variable domain" of light and heavy chains.

U.S. Patent No. 4,704,692 to Ladner (cited on page 28 lines 5-13 of the instant application) teaches that recombinant methods can be used to express unique fragments of immunoglobulins in which terminal amino acids at the N- or C-terminus of the variable region of light or heavy chains are removed as part of the strategy for linking the chains with a peptide linker to form a single chain Fv fragment. Such antibody fragments would be immunologically active while comprising less than a full length variable domain and no constant domains.

Schwartzbaum et al. (Eur. J. Immunol., vol. 19(60, 1015-1023; 1989; attached as Exhibit A) used molecular biology techniques to construct IgE antibodies with deletions in either the $C_{\varepsilon}4$ and $C_{\varepsilon}3$ constants domains (see abstract). Similarly, Bettler et al. (PNAS, 86:7118-7122, 1989; attached as Exhibit B) describes preparation of a large number of IgE constant domain deletion mutants (see Fig. 2).[1]

The existence of an adequate written description for the present claims is also consistent with the PTO's recently proposed "Revised Interim Guidelines for the Examination of Patent Applications Under the 35 U.S.C. § 112(1) 'Written Description' Requirement" ("Revised Interim Guidelines"; 64 Fed. Reg. 71427, Dec. 21, 1999). The Revised Interim Guidelines state that determining whether an inventor is in possession of the claimed invention "is a conclusion reached by weighing many factual considerations, which "include the level of skill and knowledge in the art, partial structure, physical and/or

[1] These mutants were expressed as Fc fragments.

chemical properties, functional characteristics alone or coupled with a known or disclosed correlation between structure and function, and the method of making the claimed invention." As already discussed, extensive structural and functional information and correlation of structure with function was known about immunoglobulins. In addition, the level of skill in the art was high as indicated by the extensive publication history in antibody engineering, much of this knowledge being more than 20 years old. Finally, the patent specification contains an extensive description of how to make and use the claimed invention. Thus, the factual considerations in the instant case clearly support that an adequate written description exists in the present case.

Thus, in view of the all the above, it is respectfully submitted that the phrase "at least a portion of the heavy chain constant region" finds written description support in the instant specification. Accordingly, the rejection under § 112, first paragraph, is without basis and should be withdrawn.

REJECTION UNDER 35 U.S.C. § 102 OVER STOLLE

The rejection of claims 13, 15-27, 29-65 and 83-91 under 35 U.S.C. § 102(b) as being allegedly anticipated by Stolle et al., (U.S. 4,748,018) is respectfully traversed. The rejection over Stolle et al. has been maintained despite the admitted fact that the reference does not teach or suggest the claim limitation that the antibody be obtained from transgenic plant cells. The examiner asserts without citation to any law or rule that the plant production limitation carries no weight unless Applicants demonstrate that the antibody produced in plants is different from that produced in chickens by Stolle. However, MPEP § 2116.01 requires that "[a]ll the limitations of a claim must be considered when weighing the differences between the claimed invention and the prior art in determining obviousness of a process or method claim." MPEP 2116.01 (emphasis added); see also unpublished BPAI decision in In re Vandenbergh et al. WL1771384 (BAPI 1996), (applying the "all limitations" requirement of MPEP section 2116.01 to a rejection based on anticipation; copy attached as Exhibit C). This law reflects a long standing requirement by the Federal Circuit that a determination of claim novelty requires a finding that "each and every limitation is found either expressly or inherently in a single prior art

reference." *Celeritas Techs. Inc. v. Rockwell Int'l Corp.*, 150 F.3d 1354, 1360, 47 USPQ2d 1516, 1522 (Fed.Cir.1998).

It is noted that Applicants have amended the preamble of the independent claims to further emphasize the method by which antibody is produced in the purpose of the claim (i.e., passive immunization). It is respectfully submitted, therefore, that unless the Examiner takes the position that the claims at issue are something other than a method or process claim (e.g., a product by process claim), she must acknowledge the materiality of the plant production limitation and withdraw the rejection over Stolle.

REJECTION UNDER 35 U.S.C. § 103 OVER DÜRING AND GOODMAN IN VIEW OF STOLLE

The rejection of claims 13, 15-27, and 29-91 under 35 U.S.C. § 103(a) as being allegedly unpatentable over a doctoral dissertation by Klaus During ("the During dissertation") and Goodman (U.S. 4,956,282), in view of Stolle et al, is respectively traversed. As a preliminary matter, it is pointed out that rejection over claims 66-82 is in error because the examiner has already deemed these claims withdrawn from consideration.

The examiner refers applicants to the Office Action filed August 13, 2001 as the basis for the current rejection. Applicants will address the deficiencies of the present rejection under the separate headings below.

1. **The rejection fails because it is improperly based a requirement that Applicants demonstrate that antibody expressed in plants is different from antibody in the prior art.**

The Office of August 13, 2001, which is indicated by the examiner to form the basis of the current rejection, is based on a requirement for Applicants to show that the antibodies produced by plants are structurally different from those of the prior art. As discussed above, in view of MPEP 2116.01, this is a misunderstanding of the law. All

limitations must be considered with method or process claims. Clearly, the rejection is deficient on its face because the examiner has given no weight to the limitation that the antibody be produced in plants. The claims are materially limited by the method of making the antibody, rendering this requirement that the antibody be different from the prior art as wholly irrelevant to patentability of the claims.

2. The rejection fails because one of ordinary skill would not have believed that During had successfully produced antigen-specific immunoglobulin in plants

Further review of the Office action of August 13, 2001 indicates that the Examiner based the rejection (and continues to do so) on the alleged fact that During was successful and that commercial success is not a standard for obviousness. However, this view has been directly refuted by the declaration of Richard Lerner, Ph.D., previously of record in the case. Applicants refer the Examiner to the declaration itself and Applicant's extensive discussion of this topic in the Amendment filed March 14, 2002. As discussed previously by Applicant, Lerner concludes that a person skilled in the art of immunology or protein expression, circa 1988/1989, would not have reasonably believed the assertion of the During dissertation that plant cells could be used to process and assemble an antigen-specific immunoglobulin. Lerner declaration, ¶ 22. Lerner bases this opinion on During's failure to perform critical controls to support his conclusions and to explain his inconsistent results. Also, the Ac38 antibody which underlies virtually all of the support for During's assertion cannot be used, according to Lerner, to prove that NP antigen specific binding was present in plant cells. Lerner declaration, ¶ 22. Thus, even if During had done the proper antigen inhibition controls, much more would have been needed, according to Lerner, to overcome the prejudice in the art. *Id.*

There can be no doubt that Dr. Lerner rejects the notion that During's work would have been considered successful. In fact, the undersigned has understood from a previous discussion in a related case with the examiner that the Examiner accepts Lerner's analysis of During's work. It was acknowledged that During may have produced a light chain from his dual chain vector, but there was no convincing support for heavy chain expression or assembled antibody in During's plant cells. In fact, no challenge to any of

Lerner's analyses or conclusions have been raised by the Examiner in this or in related cases.

The examiner has disregarded Lerner's challenge to During by alleging that the declaration is not commensurate in scope with the claims. Although this is incorrect as will be addressed below, it, in any event, misses the point. **Lerner is an analysis of all that is in During, so if During is relevant to the rejection, then so must Lerner be relevant. Likewise, if Lerner is off base (as the examiner mistakenly alleges), then During also must be similarly off base.** The examiner cannot take inconsistent positions in the same rejection.

3. The rejection fails because it is based on the mistaken belief that the claims are not directed to a processed and fully assembled immunoglobulin

The Office action dated November 19, 2002 attempts to disregard Applicants arguments and the analysis in the Lerner Declaration by asserting that objections to During "directed to the issue of technical difficulty of producing fully assembled antibodies in plants" are not commensurate with the claims since they "read on fragments of the fully assembled molecule."

First, it is respectfully pointed out that the claims do not read on any and all fragments of a full length antibody as stated by the Examiner. The claims read only on antigen-specific antibody fragments that have a two chain structure. In this regard, Applicants offer the declaration of Dr. Andrew Hiatt, attached herewith, to demonstrate conclusively that an antigen-specific immunoglobulin fragment requires processing and assembly as does the full-length immunoglobulin from which the fragment is derived. Hiatt declaration, ¶¶ 3-4. Thus, it cannot be said that the claims are not commensurate in scope with the arguments and support that address During's problems because such problems are applicable to both the full length immunoglobulin and antigen-specific antibody fragments. Full weight should be given, therefore, to Applicants arguments and the analysis of the Lerner declaration with respect to the deficiencies of the During dissertation.

4. The rejection fails because it is based on the mistaken belief that the findings of the Lerner declaration are allegedly not commensurate with the scope of the claims.

As already mentioned, the instant claims require that the administered immunoglobulin be formed by heavy and light chain polypeptides that are "processed" leading to an "antigen-specific" immunoglobulin. Also discussed was that heavy and light chain must properly assemble to form an antigen specific immunoglobulin, whether such antibodies are full-length or are antibody fragments with antigen specificity (e.g., Fab', Fv etc.). This view is well supported in the scientific literature as addressed in the Hiatt declaration. The Hiatt declaration further demonstrates that the prejudice in the art against producing antigen-specific immunoglobulin in plants, as described by Lerner (and affirmed by Hiatt), applies to both a full length antigen-specific antibody and its dual chain antigen-specific antibody fragment. Hiatt declaration, ¶ 5. Thus, it cannot be said that the prejudice in the art discussed in the Lerner declaration is not commensurate in scope with the claims. Full weight should be given, therefore, to Applicants arguments and the analysis of the Lerner on this point.

5. The rejection fails because there is no basis to support that the combination of cited art provides a reasonable expectation of success.

Applicants have reviewed the Office Action and the alleged basis for a reasonable expectation of success for the combination of cited art. As best as Applicants can determine, the Examiner's argument for expectation of success is that both Goodman and During teach the expression of biologically active antibodies in transgenic plant cells, and that it would have been reasonable to combine their teachings with Stolle which teaches passive immunization albeit with conventionally made antibodies. As will be shown below, this reasoning is in conflict with the facts and with positions taken by the examiner.

First, as already discussed and extensively addressed by Dr. Richard Lerner, the During dissertation is not credible and its findings would not have been accepted by those skilled in the art. In fact, this view has not been refuted or even challenged by the examiner. Thus, there simply cannot be a reasonable expectation of success when one of the references is a failure.

Furthermore, as argued many times by Applicants, Goodman's one sentence assertion that immunoglobulins can be produced amounts to nothing when viewed in light of the prejudice in the art and During's failure. Goodman's passing reference to express an immunoglobulin in plants does not come close to describing or meeting the requirements of an enabling disclosure.

All that Goodman really teaches is expression of gamma interferon in plants. Gamma interferon is not a two chain protein, nor an immunoglobulin or a protein even related to an immunoglobulin. Furthermore, the Patent Office has taken positions that contradict its position on Goodman. This has occurred in this and related cases where restriction requirements have been issued and enforced on the basis that the various forms of immunoglobulin are non obvious over each other. See e.g. U.S. serial no. 09/512,736. If the various forms of immunoglobulins are patently distinct and do not render each other obvious, it cannot be said that Goodman, who only demonstrates expression of interferon in plants, carries any weight in rendering obvious the claims.

6. Conclusion

Applicants have endeavored to fully address all arguments upon which the instant obviousness rejection is based. Various issues raised by the Examiner have been fully addressed. It is submitted that any one of these deficiencies is fatal to the rejection. However failure on all five bases demonstrates that the rejection is without basis and should be withdrawn.

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is urged to contact the undersigned by telephone to address any outstanding issues standing in the way of an allowance.

Respectfully submitted,

Date: February 19, 2003

By _____

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

13. (Amended four times) A method of passively immunizing a human or non-human animal subject against a preselected antigen **by administering an immunoglobulin produced by transgenic plant cells**, said method comprising administering to said subject a prophylactic amount of a formulation comprising an antigen-specific immunoglobulin molecule which specifically binds to said preselected antigen or immunologically active fragment of the antibody, said formulation obtained by processing plant cells containing nucleotide sequences encoding an immunoglobulin heavy chain polypeptide and an immunoglobulin light chain polypeptide wherein said nucleotide sequences also encodes a leader sequence for each polypeptide; and antigen specific immunoglobulin product encoded by said nucleotide sequences, wherein each leader sequence forms a secretion signal that is cleaved from each of said immunoglobulin heavy chain and light chain polypeptides following proteolytic processing.

41. (Amended two times) A method of passively immunizing a human or non-human animal subject against a preselected antigen **by administering an immunoglobulin produced by transgenic plant cells**, said method comprising administering to said subject a formulation comprising a prophylactic amount of an antigen-specific immunoglobulin, said formulation obtained by processing plant cells containing nucleotide sequences encoding an immunoglobulin heavy chain and an immunoglobulin light chain wherein said nucleotide sequences also encode a leader sequence for said heavy chain and said light chain and wherein each leader sequence forms a secretion signal that is cleaved from each of said immunoglobulin heavy chain and light chain polypeptides following proteolytic processing.

83. (Amended) A method of passively immunizing a human or non-human animal subject against a preselected antigen **by administering an immunoglobulin produced by transgenic plant cells**, said method comprising:

(a) preparing plant cells containing

[~~(i)~~] nucleotide sequences encoding a dual chain immunoglobulin product comprising an immunoglobulin heavy chain and an immunoglobulin light chain wherein said nucleotide sequences also encode a leader sequence

for each of said heavy chain and light chain, and antigen-specific immunoglobulin encoded by said nucleotide sequence, wherein the leader sequence forms a secretion signal that is cleaved from each of said immunoglobulin light and heavy chain following proteolytic processing; [~~or~~

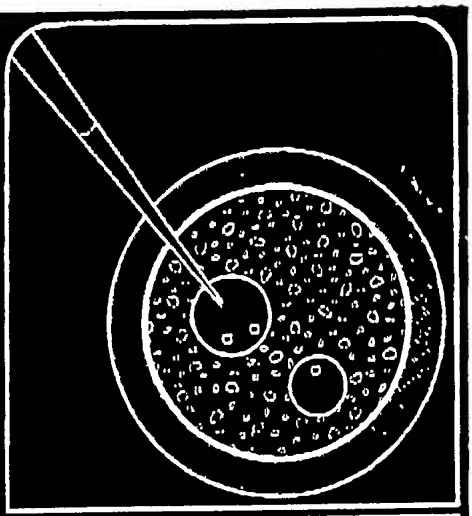
~~(ii) nucleotide sequences encoding a single polypeptide immunoglobulin comprising an immunoglobulin heavy chain and an immunoglobulin light chain and further containing nucleotide sequence encoding a leader sequence for said polypeptide, and antigen-specific single polypeptide immunoglobulin encoded by said nucleotide sequence wherein the leader sequence forms a secretion signal that is cleaved from said polypeptide following proteolytic processing;~~]

(b) isolating antigen specific immunoglobulin from the plant cells; and

(c) administering to said subject a prophylactic amount of said antigen specific immunoglobulin.

Exhibit A

EUROPEAN JOURNAL OF IMMUNOLOGY 5/1989



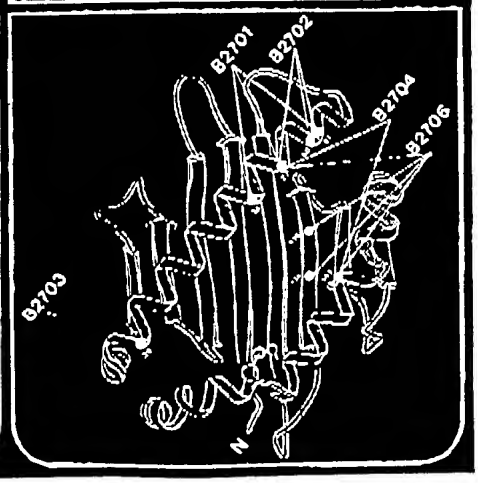


EXHIBIT A

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Mapping of murine IgE epitopes involved in IgE-Fc_ϵ receptor interactions*

The generation of anti-IgE monoclonal antibodies has permitted the identification of various serological epitopes on the IgE molecule. The relationship of the sites on IgE recognized by such antibodies to the Fc epsilon receptor ($Fc_\epsilon R$) interaction site has been determined using cross-inhibition studies. However, interpretation of this type of experiment is limited by problems of steric hindrance. Thus, to accomplish precise mapping on the IgE molecule of the $Fc_\epsilon R$ interaction site and the binding sites of various anti-IgE mAb, we employed site-directed mutagenesis of the IgE heavy chain gene.

To this end we have constructed and expressed a recombinant murine constant ε heavy chain (C_ϵ) gene bearing a (4-hydroxy-3-nitrophenyl)acetic acid (NP)-binding V_H region. Several site-specific mutants in the $C_\epsilon 3$ and $C_\epsilon 4$ domains of this recombinant C_ϵ gene were prepared and expressed by transfection into the light chain-producing J558L myeloma cell line. The resulting IgE antibodies were tested for binding to mast cells and to various anti-IgE mAb.

The mutants produced include a proline to histidine point mutant at amino acid residue 404 in the $C_\epsilon 3$ domain, a mutant with a truncated $C_\epsilon 4$ domain, a mutant with a 45 amino acid deletion in the carboxy end of $C_\epsilon 3$, and a chimeric human C_ϵ in which the human $C_\epsilon 3$ was replaced by the homologous mouse $C_\epsilon 3$ domain. These mutants have permitted the localization, to the $C_\epsilon 3$ domain, of the epitopes recognized by the 84.1C and 95.3 anti-IgE mAb. The 84.1C mAb recognizes a site on IgE which is identical or very close to the $Fc_\epsilon R$ binding site, and 95.3 recognizes a site on IgE which is related, but not identical to the $Fc_\epsilon R$ binding site. The antigenic determinant recognized by the 51.3 mAb, which is inefficient at blocking the IgE-$Fc_\epsilon R$ interaction, has been mapped to the $C_\epsilon 4$ domain. When tested for binding to the $Fc_\epsilon R$ on RBL-2H3 cells, the point mutant bound to the $Fc_\epsilon R$ with twofold reduced affinity, while the $C_\epsilon 3$ deletion mutant and the mutant truncated in $C_\epsilon 4$ lost all receptor binding activity. These data suggest that the $Fc_\epsilon R$ binding site can be assigned to the third C region domain, and that the fourth domain, while not directly involved in $Fc_\epsilon R$ binding, may play a role in the formation of the H_2L_2 tetrameric IgE molecule, and in stabilizing the conformation of IgE required for $Fc_\epsilon R$ binding.

1 Introduction

IgE antibodies are the class of antibody responsible for mediating the allergic response. Mast cells bear an Fc_ϵ receptor ($Fc_\epsilon R$) which is able to bind to IgE with high affinity. Antigen binding causes the cross-linking of the mast cell-bound IgE, triggering a series of responses in the mast cell leading to degranulation and the release of histamine and other mediators of allergy.

[I 7408]

Much work has been done in an attempt to identify the site on the IgE molecule which interacts with the $Fc_\epsilon R$. Most of the data available to date implicate the third constant (C) region domain, and the interface between the second and the third domain of IgE, in receptor binding. Experiments by Pérez-Montfort and Metzger [1] have shown that a region in the cleft between the second and third C region domains is protected from proteolysis when bound to the $Fc_\epsilon R$. Burt et al. [2, 3] made synthetic peptides corresponding to regions of the rat IgE molecule thought to be accessible to the external environment. One of these peptides, P129, corresponding to residues 414–428 in the third C region domain ($C_\epsilon 3$), (residues 401–415 using the numbering system of Liu [4], the convention adopted in this report) inhibits the binding of IgE to the $Fc_\epsilon R$ on a rat mast cell line (RBL-2H3). The binding of P129 is about 1000-fold less efficient on a molar basis than the binding of intact IgE.

In an analogous set of experiments, Robertson and Liu [5] have prepared antisera against peptides corresponding to regions of the mouse second, third, and fourth C region domains. Most of these antisera reacted against native IgE and showed varying reactivities against mast cell-bound IgE. One anti-peptide antibody, made against a peptide from $C_\epsilon 3$, "E-peptide-3", bound well to free IgE, but not to cell-bound IgE. This peptide may therefore represent a site sterically close to the epitope on IgE recognized by the $Fc_\epsilon R$. Interestingly, anti-

* This research was supported by a grant from the National Council and Development, Israel, and the European Economic Community; Z.E. is an incumbant of the Marshall and Renette Ezralow Chair in Chemical and Cellular Immunology.

+ Fellow of the Jane Coffin Childs Memorial Fund for Medical Research.

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Abbreviations: $Fc_\epsilon R$: Receptor for the constant region of IgE **mAb**: Monoclonal antibody(ies) **NIP**: (4-Hydroxy-3-iodo-5-nitrophenyl)acetic acid **NP**: (4-Hydroxy-3-nitrophenyl)acetic acid **ELISA**: Enzyme-linked immunosorbent assay **FgG**: Fowl γ-globulin **PBS**: Phosphate-buffered saline

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sera raised by Robertson and Liu against a peptide overlapping (by eight residues) Stanworth's P129 peptide, were able to bind cell-bound IgE. This led them to the conclusion that the site corresponding to this peptide is not involved in $Fc_\epsilon R$ binding.

Helm et al. [6] have generated recombinant peptides containing stretches of the human $C_\epsilon 2$, $C_\epsilon 3$ and $C_\epsilon A$ domains. They found that a peptide containing 76 amino acids spanning the $C_\epsilon 2$-$C_\epsilon 3$ junction is able to bind to the $Fc_\epsilon R$ on mast cells with an affinity close to that of myeloma IgE. In addition, monomeric fragments were produced which still bound to the $Fc_\epsilon R$, indicating that a heavy (H) chain dimer is not necessary for $Fc_\epsilon R$ binding. Nevertheless, it is not clear whether an analogous region at the $C_\epsilon 2$-$C_\epsilon 3$ junction, containing the $Fc_\epsilon R$ binding site, is present on rodent IgE.

A number of anti-IgE monoclonal antibodies (mAb) have been generated in our laboratory [7, 8]. 84.1C, 95.3 and 51.3 define three separate groups of determinants on the C_ϵ region. All three of these mAb block, to varying degress, the binding of IgE to the $Fc_\epsilon R$ on mast cells. However, only the 84.1C antibody is unable to bind to cell-bound IgE. This suggests that 84.1C recognizes an epitope identical to, or closely associated with the $Fc_\epsilon R$ binding site on IgE.

In order to reconcile some of the data on IgE-$Fc_\epsilon R$ interaction site (s), and to identify the sites on the IgE molecule recognized by our anti-IgE mAb, we constructed a panel of IgE mutants. Testing of these mutant IgE for their ability to bind to mast cells and to our anti-IgE mAb, should help create a map of various epitopes on the murine IgE molecule involved in the $Fc_\epsilon R$ binding.

2 Materials and methods

2.1 Plasmids and vectors

The pSV2-V_H6 expression vector (Fig. 1A) contains the complete rearranged V_H gene of an anti-NP (4-hydroxy-3-nitrophenyl)acetic acid antibody of the NP^b family [9], a gift of Dr. Doug Rice. It is based on the pSV2*gpt* vector [10], a mammalian expression vector containing selectable markers for growth in both bacteria (β-lactamase gene) and mammalian cells (*gpt* gene). The pA-6 vector consists of the pBR322 plasmid into which a mouse rearranged genomic IgM H chain was introduced. The pBR-C_ϵ plasmid [11], containing a genomic clone of the mouse IgE H chain cloned into the pBR322 plasmid, was a kind gift of Dr. T. Honjo (Fig. 1B).

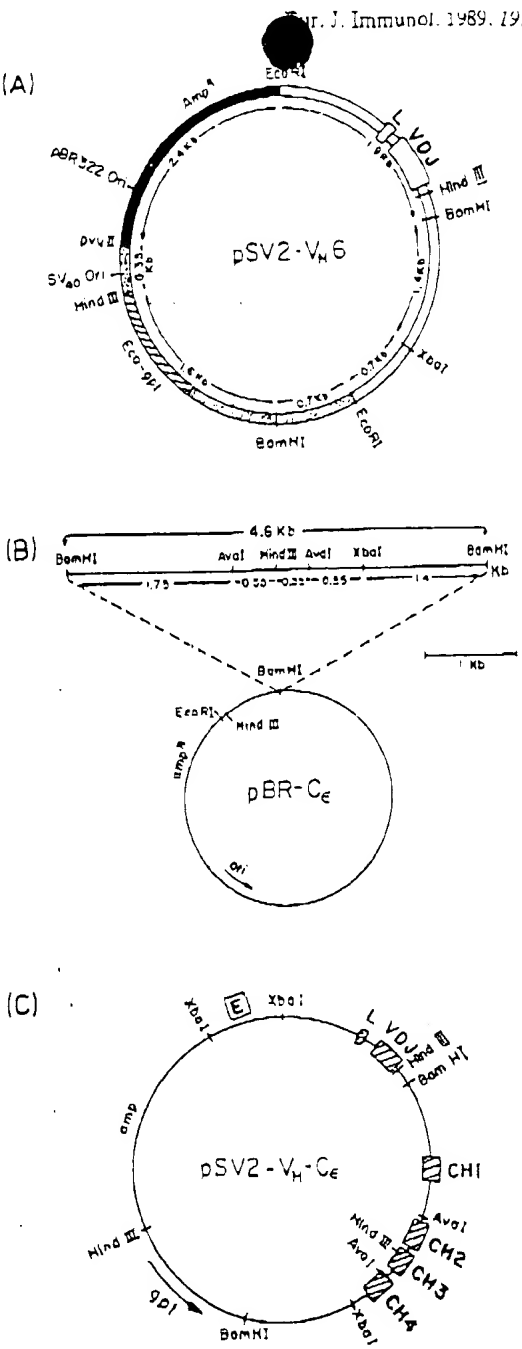


Figure 1. Schematic diagram of: (A) the pSV2-V_H6 plasmid based on the pSV2*gpt* vector [10], into which the rearranged VDJ genomic segment encoding an anti-NP [9] V_H chain was inserted. (B) The pBR-C_ϵ plasmid containing a segment of genomic DNA encoding all four C_ϵ exons [11] inserted into the pBR322 plasmid. (C) The pSV2-V_H-C_ϵ plasmid, composed of the pSV2-V_H6 vector (A), into which the C_ϵ gene (from pBR-C_ϵ) and an Ig enhancer sequence [E] were inserted flanking the rearranged VDJ segment.

2.2 Molecular biology techniques

Plasmid preparations, digestions with restriction enzymes, separation of DNA fragments, ligations, bacterial transformations and screening of bacterial colonies were all performed by standard techniques [12]. Restriction enzymes and DNA modifying enzymes were purchased from New England BioLabs (Beverly, MA), Bethesda Research Labs. (Bethesda, MD), IBI (New Haven, CT), and Pharmacia (Uppsala, Sweden). Sequencing reactions were carried out by the dideoxy method, after subcloning into the pGEM-3 vector, using the kit supplied by Promega (Madison, WI).

Site-specific mutagenesis was carried out by heteroduplex formation as described [13] with slight modifications. An aliquot of the PBR-Cε plasmid was digested with SalI, and blunt-ended; a second aliquot was digested with Eco RV and the 6.7-kb band was purified. Approximately 100–200 ng of each fragment was mixed in high-salt restriction buffer [12] together with 8 pmol kinased mutagenized oligomer (see Sect. 3 for sequence), boiled for 3 min and allowed to gradually reanneal. Heteroduplexes were filled in whith DNA polymerase and ligase, and the resulting product was extracted with phenol,

ethanol precipitated and used to transform competent bacteria (MC1061 strain).

2.3 Transfections and cell culture

RBL-2H3 cells [14], obtained from Drs. D. and T. Jovin, were grown in minimum Eagle's medium with the addition of 10% fetal calf serum (BioLab Ltd., Jerusalem, Israel), L-glutamine (Biological Industries, Kibbutz Beth Haemek, Israel), combined antibiotics (BioLab Ltd.) and fungizone (amphotericin, Biological Industries). J558L myeloma cells [15] (a gift of Dr V. T. Oi), were grown in RPMI medium with 10% fetal calf serum (FCS), L-glutamine, and combined antibiotics (referred to as J558L medium).

Transfections were carried out by electroporation [16] or protoplast fusion [17]. For electroporation, 20 μg of purified plasmid was mixed with 2×10^7 J558L cells in phosphate-buffered saline (PBS) and subjected to a 1.8 kV discharge with an Isco (Nebraska, CO) power supply model 494. Cells were put into regular J558L medium for 24–48 h reovery and then transferred to selective medium.

Protoplast fusion was done as described [17]. Protoplasts prepared from 25-ml bacterial culture, containing the plasmid of interest were fused with polyethylene glycol (PEG) 1550 (Serva, Heidelberg, FRG) to 2×10^7 J558L cells. The cells were plated in 24-well plates at 10^5/ml and cultured in J558L medium in the presence of antibiotics [100 μg/ml kanamycin or gentamycin (Sigma, St. Louis, MO)], to kill any remaining viable bacteria. Selective medium was added at 24–48 h and clones were visible in 2–3 weeks. Selective medium contained regular J558L medium with the addition of 1 μg/ml mycophenolic acid, 250 μg/ml xanthine and 15 μg/ml hypoxanthine (Sigma).

2.4 Immunochemical reagents

Rabbit anti-mouse IgE was made in our laboratory by immunizing rabbits with several mouse mAb of the IgE class. Antisera were preadsorbed on normal mouse serum-coupled Sepharose and then further purified on an IgE-Sepharose affinity column. These antibodies recognize the C_ε chain and also react with λ1 light (L) chains which are present on the SPE-7 monoclonal IgE used for immunization. Biotinylation was done by the method of Kendall et al. [18]. NIP-protein conjugates were made by the method of Brownstone et al. [19] and coupled to Sepharose 4B following cyanogen bromide activation.

2.5 IgE purification

Lysates were produced from non-secreting mutants (AFE), by suspending cells in PBS, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride (Sigma), 5 mM iodoacetamide and 0.2 U/ml aprotinin (Sigma). Cell suspensions were lysed either by freeze-thawing, sonication or the addition of 1% Nonidet-P40 (NP40).

Anti-NIP [(4-hydroxy-3-iodo-5-nitrophenyl)acetate] IgE antibodies were purified on NIP-ovalbumin (OVA)-Sepharose columns. J558L transfectoma supernatant (200–500 ml) or 10 ml of cell lysate from $\sim 10^8$ cells, were passed over NIP-OVA-Sepharose columns. The columns were then washed with PBS containing 0.5% NP40 and then extensively washed with PBS. Elution was carried out with 5 M $MgCl_2$. Eluates were dialyzed extensively against PBS, aliquoted and frozen.

2.6 Immunoassays for IgE anti-NIP antibodies

IgE anti-NIP antibodies were detected by one of several variations of the enzyme-linked immunosorbent assay (ELISA) or radioimmunoassays (RIA) described here. For the RIA, 96-well polyvinylchloride V-shaped wells (Cooke Labs., Alexandria, VA) were coated with 50 μl antigen solution (10 μg/ml NIP_9DNP_6OVA), or anti-IgE, overnight at 4 °C. Plates were washed with PBS containing 0.1% bovine serum albumin (BSA), and blocked with PBS containing 1% BSA to reduce nonspecific binding. IgE preparations (usually 50 μl transfectoma supernatant) were then added for 2 h at room temperature, followed by ^{125}I-labeled anti-IgE. Wells were washed, cut apart and counted in a gamma counter.

For ELISA assays, flat-bottom ELISA plates (Nunc, Roskilde, Denmark) were coated with either 10 μg/ml NIP-fowl γ-globulin (FgG), or a 1 : 10 dilution of serum-free supernatant from hybridoma cells secreting anti-mouse IgE mAb [7, 8], diluted in 0.06 M sodium carbonate buffer, pH 9.5. After overnight incubation at 4 °C, the plates were washed in PBS containing 0.05% Tween, and dilutions of IgE-containing transfectoma supernatants, or purified antibody were added, and the plates were incubated for 2 h at 37 °C. Plates were then washed and a 1 : 500 dilution of biotinylated rabbit anti-mouse IgE was added for 2 h. After washing, plates were treated for 30 min with avidin-coupled peroxidase (Bio-Makor, Rehovot, Israel), washed and peroxidase substrate was added. Alternatively, for the anti-NIP ELISA, after incubation with the IgE samples, plates were treated with a 1 : 1000 dilution of peroxidase-conjugated anti-mouse Fab (Bio-Makor). Peroxidase substrate was added after washing. The substrate used was 2,2′-azino-bis(3-ethylbenzthiazoline 6-sulfonic acid) (Sigma), dissolved at 1 mg/ml in 28 mM citric acid, 44 mM Na_2HPO_4, 0.3% H_2O_2. Assays were read in an automatic ELISA reader (Titertek Multiskan; Flow Labs., Rockville, MD) at 620 nm.

2.7 Binding of iodinated IgE or RBL-2H3 cells

Purified IgE derived from transfectomas was iodinated using the chloramine-T method. Protein (40–100 μg) in 100 μl PBS was reacted with 0.5–1.0 mCi (= 18.5 37 MBq) of ^{125}I and 10 μl of chloramine T (2 mg/ml) for 2 min. The reaction was stopped with 20 μl of sodium disulfide (2 mg/ml) and the mixture chromatographed on a 7-ml Sephadex G-25 column in the presence of 1% BSA, KI, and hemoglobin solution to color the protein peak. The first radioactive peak was collected, and incorporation of iodine checked by trichloroacetic acid precipitation.

For binding studies, 10^6 RBL-2H3 cells/tube were mixed with dilutions of radiolabeled IgE, in 250 μl Tyrode's buffer [20] with 1 mM EDTA. For competition assays, cold IgE competitor was added and the mixture incubated for 2 h before the addition of the radiolabeled IgE. After an additional 2-h incubation with shaking at 4 °C, 50-μl aliquots were removed in

triplicate and layered over horse serum in microtubes. The tubes were centrifuged in a microfuge for 30 s, supernatant was aspirated and pellets were counted in a gamma counter. An additional 50-µl aliquot from each sample was counted to determine total counts added. Direct binding data were analyzed by Scatchard analysis and linear least square regression.

2.8 Degranulation assay

RBL-2H3 cells (10^6) were incubated with each IgE sample for 30 min at 37 °C. Cells were washed with Tyrode's buffer and resuspended in 1 ml Tyrode's buffer. Aliquots (100 µl) were placed in microtiter wells to which were added either 250 ng/ml NIP-FgG to measure specific release or 15 µl 10% Triton to measure maximum release, or nothing to measure spontaneous release. The plates were incubated for 30 min at 37 °C and then 15 µl of supernatant was removed from each well to measure β-hexosaminidase release, an indicator of degranulation. The supernatants removed were mixed in wells of an ELISA plate with 40 µl substrate solution [1.3 mg/ml paranitrophenyl-N-acetyl-β-D-glucosamine (Sigma) in 0.04 M phosphate/citrate buffer, pH 4.5] and incubated 1–2 h at 37 °C. To stop the reaction 0.2 M glycine, pH 10.7, was added and wells were read in an ELISA reader at 405 nm. Percent specific release was calculated as:

$$\frac{(\text{specific release} - \text{spontaneous release})}{(\text{maximum release})} \times 100,$$

where spontaneous release is release in the absence of antibody, and maximum release is the β-hexosaminidase activity obtained after 1% Triton lysis of cells.

2.9 Adsorption assay for binding to the $Fc_\varepsilon R$

As an alternative means to determine the extent of binding of the mutant IgE to the $Fc_\varepsilon R$ on mast cells, we measured the ability of RBL-2H3 cells to remove anti-NIP activity from IgE-containing preparations. Dilutions (100 µl) of purified mutant and wild-type IgE were incubated in triplicate in microtiter wells with either 100 µl PBS or 100 µl PBS containing 10^6 RBL-2H3 cells. The plates were incubated for 3–4 h at room temperature, with periodic shaking. Supernatant (100 µl) was then removed from each well, and assayed for anti-NIP activity by ELISA, on NIP-FgG-coated ELISA plates. The ratio of optical density obtained from the ELISA from IgE preparations incubated with RBL-2H3 cells *vs.* those incubated without RBL-2H3 cells gives a measure of how much IgE was removed by these cells, and thereby indicates to what extent the IgE was able to bind to these cells.

2.10 Western blots and immunodetection

IgE transfectoma lysates, cell supernatants, or purified IgE preparations were run on sodium dodecyl sulfate (SDS) polyacrylamide gels [21]. After electrophoresis proteins were transferred to nitrocellulose in 15.6 mM Tris, 120 mM glycine, pH 8.3 for 4 h at 200 mA. The nitrocellulose filters were stained with Ponceau red (Sigma) to visualize protein standards, prehybridized in PBS containing 10% skim milk for 16 h at 4 °C and then hybridized in the above buffer with ^{125}I-labeled rabbit anti-mouse IgE. Filters were washed in PBS and PBS-Tween and autoradiographed.

3 Results

3.1 Construction of the PSV2-V_H-C_ε plasmid

In order to generate IgE molecules with various mutations in the Fc portion of their C region, we constructed a vector which permitted the expression, in a mammalian cell, of a complete IgE molecule of known antigenic specificity. This was accomplished by joining the gene segment from a genomic clone encoding the mouse C_ε region with a V_H gene fragment encoding a V region with anti-NP specificity [9]. The V region encoded by this gene is heteroclitic and binds the antigen NIP with higher affinity than NP. All assays measuring hapten binding were therefore performed using NIP-conjugated rather than NP proteins. The PSV-$V2_H$-C_ε plasmid which we constructed (see below) contains the entire mouse C_ε coding sequence and an anti-NP variable region (Fig. 1C). Upon transfection into the L chain-producing J558L myeloma cell line, the ε chain encoded by the recombinant gene combines with the endogenous J558L L chain to produce an IgE antibody with anti-NP specificity. Similar constructs have been used previously by Neuberger et al. [22] to obtain chimeric IgE molecules bearing a human ε gene and a NP-binding mouse V region.

The PSV2-V_H-C_ε plasmid was constructed from the PSV2-V_H6 vector ([9] and Fig. 1A). This expression vector contains a rearranged V_H gene of anti-NP specificity [9]. The 6.25-kb fragment obtained after Bam HI digestion was ligated to the 4.6-kb Bam HI fragment of the PBR-C_ε plasmid (Fig. 1B), containing the full mouse C_ε region [11]. The vector so obtained (denoted PSV2-C_ε-E) contained the full C_ε and V_H segment but was lacking the enhancer element for Ig transcription. In order to increase the expected levels of Ig expression with this vector, the 1-kb fragment containing Ig enhancer activity, obtained fom Xba I digestion of the pA-6 plasmid, was inserted into the unique Eco RI site of the PSV2-C_ε-E vector by blunt-end ligation of filled-in ends. The vector thus obtained was called PSV2-V_H-C_ε and its structure is shown in Fig. 1C.

The PSV2-V_H-C_ε plasmid was introduced into J558L L chain-producing myeloma cells using the technique of protoplast

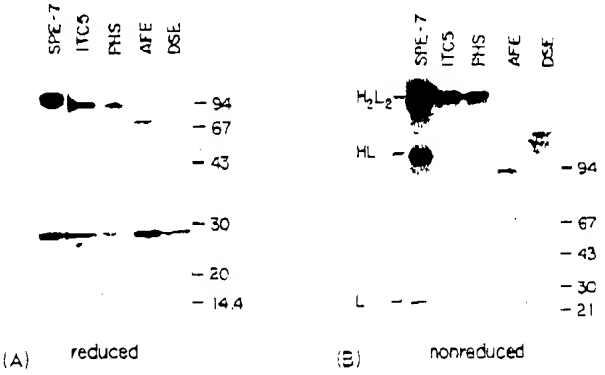


Figure 2. Immunoblot analysis of mutant and wild-type IgE antibodies. Purified IgE preparations (25–100 ng/lane) were run under reducing (A) or nonreducing (B) conditions through 10% or 5%–10% gradient acrylamide gels, respectively, electroblotted to nitrocellulose and hybridized with iodinated rabbit anti-mouse IgE antibodies. Note that in addition to the ε chain, the anti-IgE antibodies cross-react with the λ L chain.

Table 1. Properties of ITC5 recombinant IgE

Property	ITC5	SPE-7
H chain apparent molecular mass	75 kDa	75 kDa
K_a for $Fc_\epsilon R$ binding	1.2×10^8 M^{-1}	0.8×10^8 M^{-1}
$Fc_\epsilon R$ binding inhibition	2 µg/ml	2 µg/ml
Degranulation of RBL-2H3 cells	30%	28%

a) Mouse monoclonal IgE [23].
b) Determined by Scatchard analysis of binding of radioiodinated IgE to RBL-2H3 cells.
c) Concentration of IgE giving 50% inhibition of the binding of radioiodinated SPE-7 to RBL-2H3 cells.
d) % RBL-2H3 cell degranulation induced by IgE at 100 ng/ml, triggered by NIP-DNP-BSA.

fusion. Supernatants from clones surviving growth in selective medium containing mycophenolic acid were screened for IgE anti-NIP activity using a RIA (not shown) and the ITC5 clone was chosen for all further characterization. This clone secretes approximately 2 µg IgE/ml. Table 1 summarizes properties of the ITC5 IgE as compared to SPE-7 hybridoma-derived monoclonal IgE [23]. Both IgE have similar molecular weights (Fig. 2), bind with similar affinities to the $Fc_\epsilon R$ on mast cells and both are able to induce RBL-2H3 degranulation in the presence of cross-linking antigen.

3.2 Generation of vectors encoding mutant IgE antibodies

The mutated IgE antibodies that we describe in this study are depicted schematically in Fig. 3 and Table 2. They include AFE, truncated in $C_\epsilon 4$, DSE, with a 45-amino acid deletion in the $C_\epsilon 3$ domain, and PHS, with a proline to histidine point mutation in $C_\epsilon 3$. The AFE mutant was made by creating a frameshift mutation at the Ava I site in the $C_\epsilon 4$ domain (Fig. 1B, C). This frameshift caused a stop codon to be reached at amino acid 472 (position 34 of the $C_\epsilon 4$ domain). The region between the mutation site and stop codon was expected to have altered sequence due to the frameshift introduced (Fig. 3).

To generate this mutation, the PSV2-V_H-C_ϵ vector was partially digested with Ava I and the 11.85-kb linearized fragment was blunt ended with the Klenow fragment of DNA polymerase I. This was self ligated and used to transform bacteria. Restriction mapping and DNA sequencing were used to confirm that the correct Ava I site was mutated (not shown). The vector containing the AFE mutation was used to transfect J558L cells. Clones surviving mycophenolic acid selection were tested by an ELISA assay for secretion of anti-NIP antibody. Of nine clones tested from two separate transfection experiments, none secreted detectable amounts of IgE. (The sensitivity of the ELISA is approximately 5 ng/ml). It is possible that the cells can produce IgE, but that due to the gross structural alteration of IgE, they are unable to secrete IgE. We therefore produced cell lysates, using sonication, freeze-thawing or NP40 lysis. The lysates were tested for IgE anti-NIP activity by ELISA and were found to contain between < 5 and 110 ng/10^6 cells. Immunoblot analysis (Fig. 2) under reducing conditions of affinity-purified AFE mutant IgE from such lysates revealed H chains of reduced size as expected for this mutant. Electrophoresis under nonreducing conditions reveals that almost all of the antibody is present as HL, instead of the full H_2L_2 tetramer (Fig. 2).

Another deletion mutant (DSE) was constructed with a deletion of 45 amino acids in $C_\epsilon 3$ and the beginning of $C_\epsilon 4$ (amino acid residues 397–441). The delection encompasses the sequence spanned by the P129 peptide of Burt and Stanworth [2] and also includes a cysteine which participates in the $C_\epsilon 3$ intradomain disulfide bridge [4, 24]. This mutant was made by digestion of the PSV2-V_H-C_ϵ plasmid with Spe I and Eco 47III (each of which cut the plasmid at a single site), blunting the ends with mungbean nuclease, religating the large linear fragment and transforming bacteria with the product of this reaction. The structure of this plasmid was checked by restriction mapping and by hybridization to an oligomer which was complementary to the expected sequence of the junction site after ligation. Transfected clones were found to secrete antibody, but in very small amounts, 5–40 ng/ml.

A proline to histidine point mutation (PHS) was created at amino acid residue 404, using oligonucleotide-directed, site-

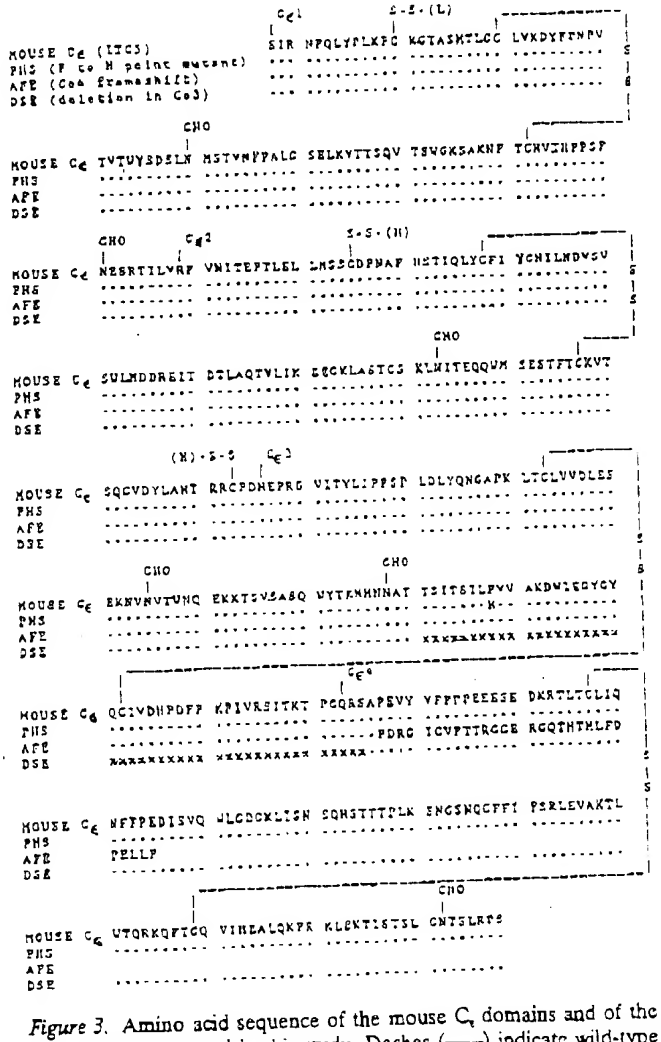
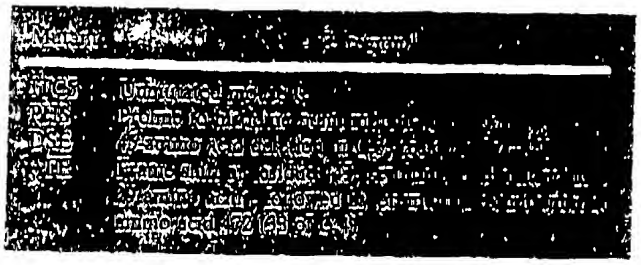


Figure 3. Amino acid sequence of the mouse C_ϵ domains and of the IgE mutants prepared in this study. Dashes (——) indicate wild-type sequence; (x) indicates deleted amino acids; (CHO) indicates glycosylation sites and (S-S) indicates intrachain disulfide bonds. S-S-(H)- and S-S-(L)- represent the interchain disulfide bonds with H and L chains. The sequence shown is based on references [4] and [23] and the one-letter amino acid code is given in [4].

Table 2. Summary of mutants produced in this study



a) All the recombinant IgE molecules listed above contain a NIP-specific mouse V_H region with a C_ε region and express the endogenous J558L mouse λ1 L chain.

specific mutagenesis [13]. This mutation is located in $C_\varepsilon 3$, in the region spanned by the P129 oligomer of Burt and Stanworth [2]. Aliquots of the PBR-C_ε plasmid were digested with Sal I and Eco RV, and the resulting digests were mixed, melted, and reannealed in the presence of a molar excess of kinased SAS3 oligomer (sequence GGC, AAC, TAC, ATG, TAG, GAT, GGA). This oligomer is complementary to the sequence encoding amino acids 401–407, with a C to A mutation which alters proline 404 to histidine. The oligomer also contains a silent mutation to increase the ease of detecting mutant *vs.* wild type. The positions of the two mutations are underlined. Mutant colonies were identified by hybridization with radiolabeled oligomer. The 4.6-kb insert corresponding to C_ε was removed by digestion with Bam HI and ligated into the PSV2-V_H-C_ε plasmid from which the wild-type C_ε sequences were removed. This plasmid (PSV-V_H-C_εPHS) was used to transfect J558L cells by electroporation. Supernatants from clones surviving mycophenolic acid selection were tested for IgE anti-NIP antibodies. The PHS clone selected secretes approximately 2 μg/ml of antibody. RNA was isolated from this clone and tested by Northern blot hybridization with the mutant oligomer for the presence of the mutated sequence. The oligomer hybridizes to PHS but not to ITC5 (wild-type) RNA (not shown). SDS gel electrophoresis of the IgE produced by the PHS mutant reveals H and L chains of the expected size, present as H_2L_2 in nonreduced gels (Fig. 2).

The preparation and expression of vectors containing the human C_ε sequences, and human-mouse chimeras will be described in a separate manuscript (A. Nissim and Z. Eshhar, in preparation).

3.3 Binding of the mutant IgE to the $Fc_\varepsilon R$ on RBL-2H3 cells

In order to identify the sites on the IgE molecule involved in the interaction with the $Fc_\varepsilon R$, the IgE mutants were tested for their ability to bind to RBL-2H3 cells, a rat mast cell line bearing high-affinity FcR for rodent IgE [14]. A number of different assays were used to measure binding. First, affinity-purified preparations of unmutated ITC5 and the IgE mutants were tested for their ability to inhibit the binding of iodinated, purified monoclonal IgE (SPE-7) [24] to RBL-2H3 cells. The inhibition curves so obtained are shown in Fig. 4A. It may be seen that the PHS point mutant is reduced approximately twofold in its ability to bind to the $Fc_\varepsilon R$, while the DSE mutant has lost all ability to compete with radioiodinated IgE for the binding to RBL-2H3 cells. The AFE mutant, which is not secreted, and was therefore purified from cell lysates, was not available in sufficient concentrations for the inhibition assay. It was therefore tested in the other binding assays described below. Since the differences in the inhibition of binding by the ITC5 wild-type IgE and the PHS mutant were small, these IgE preparations were also tested for direct binding to RBL-2H3 cells. Affinity-purified ITC5 and PHS were radioiodinated and the binding of these antibodies to RBL-2H3 cells was tested. Scatchard analysis (Fig. 4B) reveals that the PHS mutant has a 2.5-fold lower affinity for the $Fc_\varepsilon R$ than ITC5 wild-type IgE.

These results were further confirmed in two other assays. In an assay measuring the degranulation of RBL-2H3 cells, the ITC5 (wild type) and PHS (mutant) IgE antibodies, but not the DSE or AFE mutant IgE were able to induce degranulation of RBL-2H3 cells, when cross-linked on the cell surface by polyvalent antigen (NIP-FgG) (Table 3).

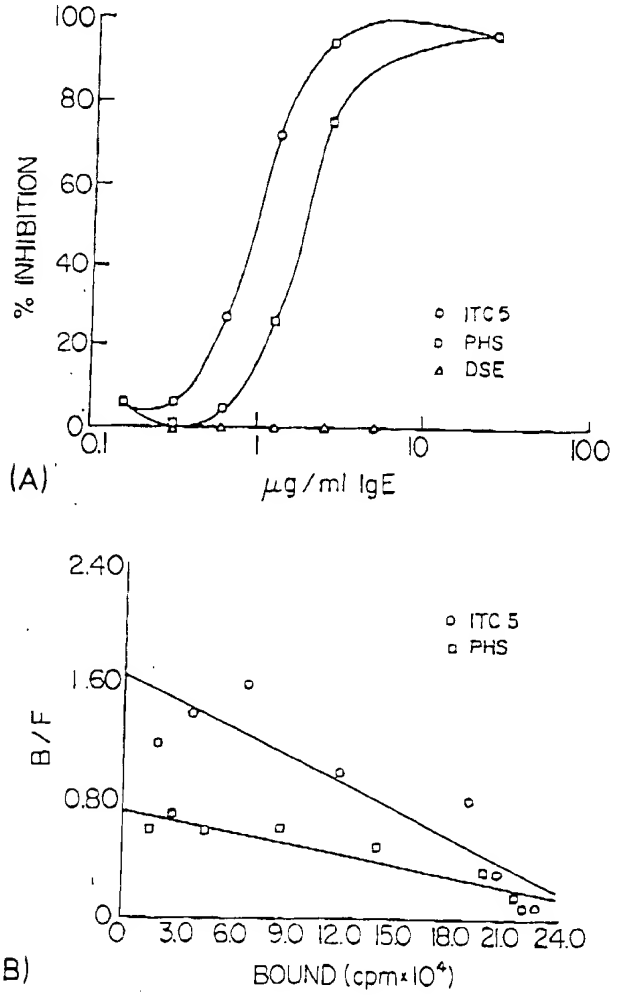
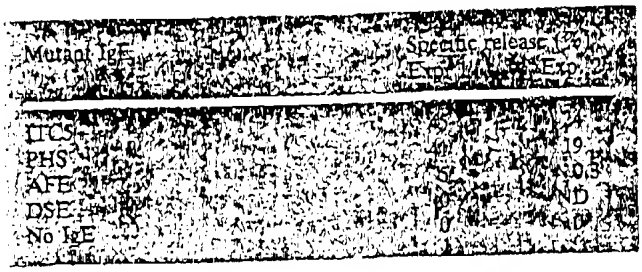


Figure 4. $Fc_\varepsilon R$ binding of mutant IgE antibodies. (A) Inhibition of binding of iodinated IgE to RBL-2H3 cells by mutant and wild-type recombinant IgE. RBL cells were preincubated with varying concentrations of ITC5 (wild type), PHS (point mutant) or DSE (deletion mutant) IgE. ^{125}I-labeled monoclonal IgE was then added and after a 2-h incubation, cell-bound and free IgE were separated and radioactivity was counted. All determinations were done in triplicate. (B) Scatchard analysis of binding of ITC5 (unmutated) and PHS, point mutant IgE. RBL-2H3 cells were incubated with several concentrations of iodinated ITC5 and PHS IgE. Unbound counts were removed by centrifugation through serum, and the cell pellet was counted. Lines represent the least squares fit, derived by linear regression analysis.

Table 3. Degranulation of RBL-2H3 cells mediated by IgE mutants



a) RBL-2H3 cells were preincubated with affinity-purified IgE preparations at 0.2 μg/ml for 30 min and washed before the addition of NIP-FgG (250 ng/well). β-Hexosaminidase release was determined as described in Sect. 2.8. Each determination was done in triplicate. Standard deviations were < 15% of ELISA readings.

Finally, mutant IgE antibodies were tested for their ability to be adsorbed by RBL-2H3 cells. Dilutions of the affinity-purified antibodies were incubated with RBL-2H3 cells to allow binding. Supernatants containing unbound IgE were tested by ELISA for anti-NIP activity. Results were plotted as the ratio between the ELISA readings obtained for adsorbed supernatants, divided by unadsorbed supernatants, *vs.* the concentration of the IgE preparation used (represented by ELISA reading). As can be seen in Fig. 5, the ratio of A_{620} "adsorbed"/A_{620} "unadsorbed" was lowest for ITC5, the wild-type IgE (best adsorbed), and higher for the PHS mutant, though some anti-NIP activity was still removed. For the AFE and DSE mutants, the ratio was close to one, indicating that the RBL-2H3 cells did not remove any of the IgE anti-NIP antibodies. Thus, we may conclude that binding of mutant IgE molecules to the $Fc_\epsilon R$ on rat mast cells follows the order: ITC5 > PHS ≫ AFE = DSE.

3.4 Recognition of mutant IgE antibodies by anti-IgE mAb

Purified mutant and wild-type IgE antibodies were next tested for their ability to bind to anti-mouse IgE mAb which have been previously described [7, 8]. ELISA assays were performed in which wells were coated with either antigen (NIP-FgG), or serum-free preparations of the anti-IgE mAb. This was followed by dilutions of purified wild-type and mutant IgE preparations. Degree of binding was determined using biotinylated rabbit anti-mouse IgE (H + L), and peroxidase-labeled avidin. Table 4 shows the results of one representative assay.

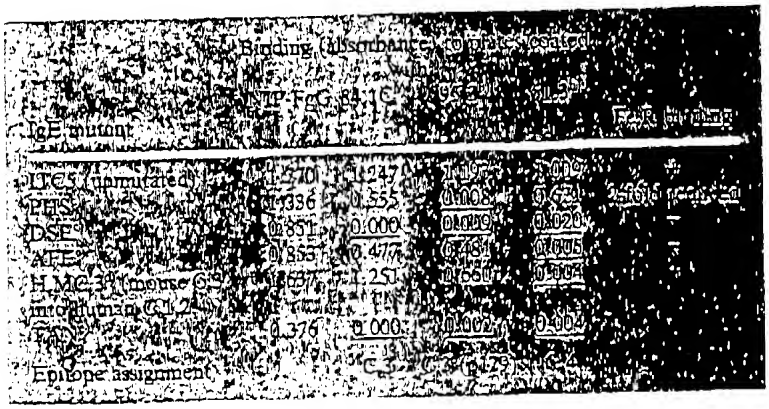
It may be seen that the PHS point mutant has lost the ability to be recognized by the 95.3 anti-IgE mAb, while it is still recognized by the 51.3 and 84.1C mAb (Table 4) and can still bind to mast cells (though with reduced affinity) (Fig. 4). The DSE deletion mutant has lost both recognition by all three mAb and the ability to bind to the $Fc_\epsilon R$ on mast cells. The AFE mutant which has a frameshift causing premature termination in the $C_\epsilon 4$ domain has reduced binding to the 51.3 anti-IgE mAb, and has lost the ability to bind to the FcR on RBL-2H3 cells. A chimeric human IgE ($H_\epsilon MC_\epsilon 3$) containing mouse $C_\epsilon 3$ (manuscript in preparation), binds the 84.1C and 95.3 mAb, but not the 51.3 mAb. "TAN" human IgE (manuscript in preparation), with the J558L L chain and mouse anti-NIP V_H region, is not recognized by any of the mAb. These data permit the assignment of the site recognized by 84.1C and 95.3 to the $C_\epsilon 3$ domain, and that recognized by 51.3 to the $C_\epsilon 4$ domain.

4 Discussion

In order to learn more about the $Fc_\epsilon R$ binding site on the IgE molecule, we have developed an *in vitro* expression system enabling the production of genetically engineered mouse IgE with anti-NP activity. The antibody so produced, represented by clone ITC5, has all the properties of authentic mouse IgE and binds with high affinity to the $Fc_\epsilon R$ on mast cells (Table 1). The plasmid encoding the ITC5 antibody was then subjected to a variety of mutagenesis procedures in order to produce IgE molecules with alterations in their Fc region. These mutants were used in binding assays to determine their ability to bind to the $Fc_\epsilon R$ on mast cells and to bind to a series of anti-IgE mAb previously generated in our laboratory [7, 8].

Four assays were employed to measure the binding of mutant or wild-type IgE to the $Fc_\epsilon R$ on mast cells: (a) the inhibition of binding of radiolabeled IgE to RBL-2H3 cells, (b) the direct binding of iodinated IgE to RBL-2H3 cells, (c) IgE-mediated degranulation of RBL cells and (d) the adsorption, by RBL-2H3 cells, of anti-NIP activity from IgE-containing solutions. These assays gave concordant results. Compared to the ITC5 wild-type IgE, the PHS proline to histidine point mutant bound the $Fc_\epsilon R$ with approximately twofold reduced affinity (Figs. 4A, B and 5). PHS mutant IgE induced almost as much degranulation as did ITC5 wild-type IgE (Table 3). Since only

Table 4. Binding of IgE mutants to anti-IgE mAb



a) Purified unmutated (ITC5) and mutated recombinant IgE were tested by ELISA for binding to various anti-mouse IgE mAb. ELISA plates were first coated with serum-free supernatant containing the various anti-IgE mAb, or with NIP-conjugated protein. IgE (4 ng) diluted in medium (DMEM and FCS) was then added to each well. After washing, the wells were treated with biotin-conjugated rabbit anti-mouse IgE, followed by an avidin-peroxidase conjugate and peroxidase substrate. Absorbance was read in an automatic ELISA reader. Variation between duplicates was always < 10% of the values read, or < 0.01 absorbance units for negative wells.
b) Anti-mouse IgE mAb.
c) See Table 2.
d) Mouse $C_\epsilon 3$ into human $C_\epsilon 1,2,3$.
e) Human IgE.

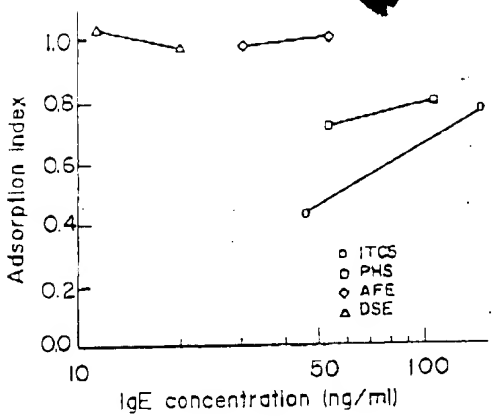


Figure 5. Adsorption of IgE by RBL-2H3 cells. Dilutions of mutant and wild-type IgE antibodies were incubated with RBL-2H3 cells for 4 h in microtiter wells. After this incubation, supernatants were removed and tested by ELISA for anti-NIP activity. Results were plotted as the adsorption index (the ratio between the ELISA readings obtained for adsorbed divided by unadsorbed supernatants) *vs.* the concentration of the IgE preparation tested. An adsorption index closer to unity indicates less adsorption of IgE by RBL-2H3 cells, and hence, less binding of the particular mutant.

a small fraction of $Fc_\epsilon R$ on RBL-2H3 cells need to be occupied in order to induce degranulation,it is not surprising that sufficient occupancy could be obtained with either ITC5 wild type or PHS point mutant IgE.

The AFE frame shift mutant, lacking a functional $C_\epsilon 4$ domain, and the DSE mutant, with a 45-amino acid deletion in $C_\epsilon 3$, are unable to bind to the $Fc_\epsilon R$ in the four assays used (Figs. 4 and 5 and Table 3). It is surprising, in light of the accumulated evidence [1–3, 5] suggesting that the binding site for the $Fc_\epsilon R$ is present on $C_\epsilon 3$, that the AFE mutant (lacking the $C_\epsilon 4$ domain) is unable to bind to the $Fc_\epsilon R$. We believe that the $C_\epsilon 4$ domain, while not containing the actual receptor binding site(s), is necessary for the IgE molecule to attain the correct conformation needed for receptor binding. The $C_\epsilon 4$ domain has been shown to be important for the dimerization of human IgE [6]. Interestingly, our AFE mutant mouse IgE which lacks most of the $C_\epsilon 4$ domain, is present inside the cell almost entirely as HL (Fig. 2). This indicates that in mouse IgE, the fourth domain may also have a role in initiating, or stabilizing H chain dimerization.

The epitopes recognized by three anti-IgE mAb, 84.1C, 95.3 and 51.3, were assigned to domains on the ε chain based on their ability to recognize the various IgE mutants tested. For example, the 95.3 mAb was shown to recognize a determinant on the $C_\epsilon 3$ domain, since it is no longer recognized by the PHS mutant, which has a single proline to histidine substitution in $C_\epsilon 3$, and is still recognized by the AFE mutant which has 96% of its $C_\epsilon 4$ domain altered or deleted. In addition, 95.3 is able to recognize the mouse-human chimeric IgE, which has only mouse $C_\epsilon 3$, while it is unable to recognize human IgE. By analogous logic, the epitope recognized by 84.1C may be assigned to $C_\epsilon 3$, and the epitope recognized by 51.3 is most likely on $C_\epsilon 4$ (Table 4).

The purified DSE mutant (45-amino acid deletion in $C_\epsilon 3$) seems to bind none of the anti-IgE mAb, including the 51.3 antibody which we have assigned to $C_\epsilon 4$. However, when DSE transfectoma supernatant was tested in a similar assay, some reaction was seen against the 51.3 and 84.1C mAb (not shown). It is possible that conformational changes in this mutant have reduced binding to these two anti-IgE mAb, and that these conformational changes in the DSE mutant are more pronounced when the mutant IgE is subjected to high-salt elution from the affinity column. Similarly, in a number of experiments, when fresh DSE supernatants were tested for binding to the $Fc_\epsilon R$ on RBL cells using the adsorption assay, some binding to the $Fc_\epsilon R$ was detected. Purified fractions of this mutant, eluted with 5 M $MgCl_2$, consistently failed to bind. Thus, the effects of the deletion on the binding to the 84.1C and 51.3 anti-IgE mAb, and on the binding to the $Fc_\epsilon R$ are likely to be conformational, rather than due to the elimination of the binding site. We have previously shown [7, 8] that the 51.3, 95.3 and 84.1C anti-IgE mAb are sensitive to IgE conformation and are unable to bind to denatured or reduced IgE.

The anti-IgE antibodies have previously been analyzed in inhibition studies to determine the relationship of the epitopes they recognize to the binding site for IgE. The 51.3 antibody inhibits poorly the binding of IgE to its receptor and is able to bind to IgE receptor complexes [7]. Thus, it seems that this antibody recognizes an epitope which is not closely related to the receptor binding site. This is consistent with our conclusion that the epitope recognized by this antibody is on the $C_\epsilon 4$ domain. The 95.3 anti-IgE mAb inhibits the binding of IgE to its $Fc_\epsilon R$ but can still bind to cell-bound IgE [7]. Thus the epitope recognized by this antibody is related, but not identical to the receptor binding site. This is also apparent from the fact that the PHS point mutant has lost, entirely, the epitope recognized by 95.3, but is still able to bind to the $Fc_\epsilon R$ on mast cells, albeit with reduced affinity. The reduced ability of the PHS mutant to bind to the $Fc_\epsilon R$ on RBL cells may be due to a direct effect of the proline to histidine mutation in destabilizing the IgE-$Fc_\epsilon R$ interaction, or could be due to conformational changes caused by this mutation which are propagated to distal sites on the $C_\epsilon 3$ domain. While more mutants will be required in order to distinguish between these possibilities, it is clear that the 95.3 binding site can be totally abolished with only a moderate effect on $Fc_\epsilon R$ binding.

The 84.1C anti-IgE mAb inhibits the binding of IgE to the $Fc_\epsilon R$ and does not recognize cell-bound IgE [8]. Thus, it seems likely that this antibody recognizes an epitope very closely related to the binding site for the $Fc_\epsilon R$. It will be interesting to see whether among further IgE point mutants which we are currently producing, one will be found which looses both the ability to bind to the $Fc_\epsilon R$ and to the 84.1C anti-IgE mAb. A good candidate for such a mutation may be a site on the "E-peptide-3" produced by Liu et al. [5]. Antibodies against this $C_\epsilon 3$ peptide behave like our 84.1C mAb in that they bind to free, but not $Fc_\epsilon R$-bound IgE, and thus seem to cross-inhibit the IgE-$Fc_\epsilon R$ interaction.

By combining the above data, a few conclusions may be reached. We have mapped to the $C_\epsilon 3$ domain the 84.1C anti-IgE mAb most closely related to the IgE-$Fc_\epsilon R$ interaction site. This determinant is not included within the P129 peptide site described by Burt and Stanworth [2]. A single proline to histidine substitution in the region spanned by the P129 peptide leads to the loss of recognition by the 95.3 mAb, which recognizes an epitope that is related, but not identical to the $Fc_\epsilon R$ binding site. However, such a mutant (PHS) retains full recognition by the 84.1C anti-IgE mAb, and exhibits only two-fold reduction in binding to the $Fc_\epsilon R$. Thus, the $Fc_\epsilon R$ binding

site is probably located in the $C_\varepsilon3$ domain, but not in the region encompassed by P129. The $C_\varepsilon4$ domain seems to be required for the formation of the H_2L_2 tetramer and, though not directly involved in $Fc_\varepsilon R$ binding, may play a role in stabilizing the conformation of IgE required for binding to the FcR. The generation of more IgE deletion and point mutants, together with mouse/human exon shuffling experiments (manuscript in preparation) should allow the precise identification of sites on the IgE molecule required for dimerization, binding to mAb and binding to the high-affinity $Fc_\varepsilon R$ on mast cells.

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Exhibit B

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Immunology

Binding site for IgE of the human lymphocyte low-affinity Fc_ε receptor ($Fc_\varepsilon RII$/CD23) is confined to the domain homologous with animal lectins

(IgE-binding factor/structure–function analysis/epitope mapping/mammalian expression vector)

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ABSTRACT The lymphocyte low-affinity receptor for IgE ($Fc_\varepsilon RII$) is involved in two seemingly unrelated processes: (*i*) promotion of general B-cell growth and (*ii*) isotype-specific IgE synthesis. To characterize domains of $Fc_\varepsilon RII$ important for effector function, we have expressed $Fc_\varepsilon RII$ mutants in mammalian cells. The results show that the IgE-binding region of $Fc_\varepsilon RII$ corresponds almost exactly to a domain of 123 amino acid residues homologous with the carbohydrate-binding domain of C-type animal lectins. With the recent demonstration that $Fc_\varepsilon RII$ binds to IgE independently of any lectin-like activity [Vercelli, D., Helm, B., Marsh, P., Padlan, E., Geha, R. S. & Gould, H. (1989) *Nature (London)* 338, 649–651], it is now clear that, in this case, the lectin module has evolved to interact with a protein rather than a carbohydrate moiety. The epitopes of several independent monoclonal antibodies that inhibit the binding of IgE to $Fc_\varepsilon RII$ are clustered within the lectin-like domain. Some of these antibodies are also known to suppress, isotype-specifically, the interleukin 4-promoted IgE synthesis from peripheral blood mononuclear cells or the spontaneous synthesis of IgE by B cells isolated from atopic donors. The epitope of MHM6, an anti-$F_\varepsilon RII$ monoclonal antibody delivering an epitope-restricted growth-promoting effect on B cells, is also located within the lectin-like domain. Thus, the lectin module of $Fc_\varepsilon RII$ not only acts as a carbohydrate-independent, isotype-specific Fc receptor but may also participate in the general regulation of B-cell growth.

IgE is the class of immunoglobulin responsible for most allergic diseases (1) and is involved in immunity against parasites (2). The effector functions of IgE are brought about by binding to specific cell surface receptors. A high-affinity receptor ($Fc_\varepsilon RI$) and a low-affinity receptor ($Fc_\varepsilon RII$) for the Fc part of IgE have been described in the human system (3–6). $Fc_\varepsilon RI$ mediates effector release from mast cells and basophils in immediate-type hypersensitivity reactions. $Fc_\varepsilon RII$, identical with the 45-kDa B-cell differentiation antigen CD23 (7, 8), is structurally distinct from the four-chain $Fc_\varepsilon RI$ molecule. $Fc_\varepsilon RII$ is expressed on many leukocytes and plays a role in isotype-specific processes such as regulation of IgE synthesis (6, 9, 10) and IgE-dependent cytotoxicity against parasites (2). $Fc_\varepsilon RII$ is also important in more general B-cell growth-regulatory pathways (5, 11–13). MHM6, an anti-$Fc_\varepsilon RII$ monoclonal antibody (mAb), has been shown to trigger the progression of activated B cells through the G_1 phase of the cell cycle (11, 12). Beyond its implication in a specific as well as in a more general B-cell regulation system, a role for $Fc_\varepsilon RII$ in cell adhesion has also been proposed (14, 15).

The cDNA for the human $Fc_\varepsilon RII$ has been cloned and functionally expressed in mammalian cells (16–18). It encodes a protein of 321 amino acid residues with an inverse membrane orientation; the N terminus is cytoplasmic and the C terminus is exposed at the cell exterior. The C-terminal half of the extracellular domain is shed from the membrane as a 25-kDa molecule as a result of proteolytic cleavage. This soluble fragment has been termed an IgE-binding factor (IgE-BF). It is found complexed with IgE in the serum of atopic patients (7) and its level is highly elevated in patients with B-cell-derived chronic lymphocytic leukemia (19). Furthermore, T cells of patients infected with human immunodeficiency virus type 1 are induced to produce IgE-BF (20).

The N terminus of $Fc_\varepsilon RII$-derived IgE-BF starts at amino acids 148–150 of $Fc_\varepsilon RII$ (16–18). The middle region of this IgE-BF shows a marked degree of homology with animal lectins (21, 22), nonenzymatic proteins that bind selectively to specific carbohydrate structures (23). Recently, it became evident that there exists in the hematopoietic system a family of adhesion receptors, including Mel-14, ELAM-1, and GMP-140, with homology to the lectin domain of $Fc_\varepsilon RII$ (24). These molecules control lymphocyte migration, probably through carbohydrate-dependent recognition events.

The present experiments were undertaken to delimit the IgE-binding domain of $Fc_\varepsilon RII$. Further, we have mapped the epitopes of several anti-$Fc_\varepsilon RII$ mAbs that have been reported to influence either the isotype-specific or the B-cell growth-promoting processes.

MATERIALS AND METHODS

Assembly of Expression Plasmid pCAL5mβDhfr for Mammalian Cells. Plasmid pSVneo2911 (25) was used to prepare a *Sal* I–*Eco*RI DNA fragment containing the ampicillin-resistance, tetracycline-resistance, and dihydrofolate reductase (Dhfr) genes. The murine cytomegalovirus (MCMV) immediate-early gene enhancer and promoter, used to drive $Fc_\varepsilon RII$ cDNA expression, are contained in the 540-base-pair (bp) *Acc* I–*Hin*dIII fragment of the simian virus 40–MCMV recombinant virus P1 (26). The 1.26-kilobase (kb) $Fc_\varepsilon RII$ cDNA insert was prepared by digesting plasmid pSVd-ER (17) partially with *Hin*dIII and completely with *Bam*HI. The 3′ half of the rabbit β-globin gene was derived from plasmid pUβ (27). Its 1.2-kb *Bam*HI–*Sal* I fragment contains the large β-globin intron and the polyadenylylation signal. All these cartridges were combined to construct the expression plasmid pCAL5mβDhfr (Fig. 1). Incompatible restriction cleavage sites were filled in with Klenow enzyme and joined by blunt-end ligation.

Construction of $Fc_\varepsilon RII$ Deletion Mutants. The *Sal* I–*Eco*RI DNA fragment from plasmid pSVd-ER (17) containing the

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Abbreviations: $Fc_\varepsilon RII$, lymphocyte low-affinity receptor for IgE; mAb, monoclonal antibody(ies); pAb, polyclonal antibodies; Dhfr, dihydrofolate reductase; IgE-BF, IgE-binding factor(s); MCMV, murine cytomegalovirus; BCGF, B-cell growth factor.

EXHIBIT B

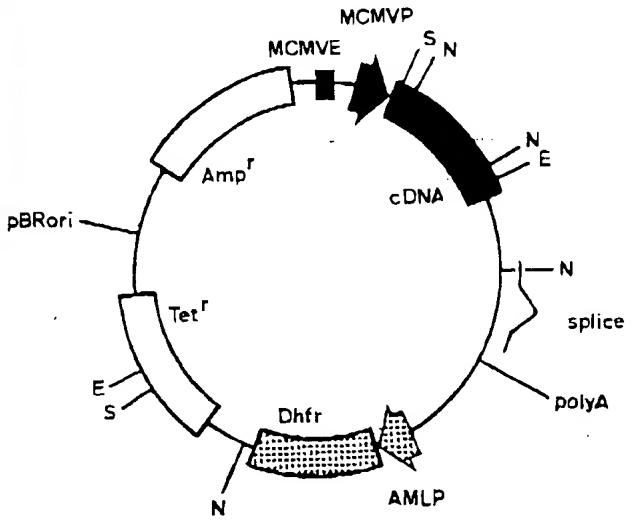

FIG. 1. Mammalian expression vector pCAL5mβDhfr. The region encoding various $Fc_\epsilon RII$ constructs is shown by a heavy black bar. cDNA expression is driven by an enhancer/promoter unit (MCMVE/MCMVP) of the MCMV (black box and arrow). The Dhfr coding sequence (stippled bar) is under control of the adenovirus major late promoter (AMLP; stippled arrow). The genes for ampicillin (Amp^r) and tetracycline (Tet^r) resistance are shown as open bars. Restriction sites indicated were used for construction of $Fc_\epsilon RII$ mutants (N, *Nco* I; E, *Eag* I; S, *Sal* I). In addition, the rabbit β-globin polyadenylylation (polyA) and splice sites as well as the bacterial pBR322 replication origin (pBRori) are shown.

entire coding region of $Fc_\epsilon RII$ was inserted into the polylinker of Bluescript M13 phagemid SK− (Stratagene). After infection with helper phages, single-stranded phage DNA carrying the cDNA insert was purified and used as template for mutagenesis. Oligonucleotide-directed *in vitro* mutagenesis (Amersham kit) employed methods recommended by the manufacturer, except that the exonuclease III treatment to remove the nonmutated strand was shortened to 20 min. All oligonucleotides used in the mutagenesis reaction were purified by PAGE prior to use. C-terminal deletion mutants were constructed by inserting the stop codon TGA into the reading frame. Internal deletions were generated by looping out the desired cDNA region in the mutagenesis reaction. To allow rapid screening, mutants were designed so as to introduce a novel restriction site into the cDNA without altering the amino acid sequence. Plasmids bearing each mutation were cleaved with *Sal* I and *Eag* I, which remove a cDNA fragment covering the base changes introduced. This fragment was ligated to two other DNA fragments (*Sal* I–*Sal* I, containing the ampicillin-resistance gene, and *Sal* I–*Eag* I, containing the Dhfr gene cassette) to restore the mammalian expression vector pCAL5mβDhfr (Fig. 1). The double mutant Δ134–160/X288 (Fig. 2) was obtained by combining the two corresponding deletion mutants. Control plasmid Δ1–298 (Fig. 2) was constructed by removing the *Nco* I–*Nco* I cDNA fragment of pCAL5mβDhfr. Sequence analysis (Sequenase kit, United States Biochemical) of the mutations was performed on the double-stranded expression plasmids with the use of synthetic oligonucleotide primers.

Antibodies Specific for $Fc_\epsilon RII$. We used a recombinant C-terminal $Fc_\epsilon RII$ fragment (amino acids 119–321) produced in *Escherichia coli* to immunize rabbits. This protein elicited an immune response against native $Fc_\epsilon RII$. The polyclonal antibodies (pAb) were purified by standard methods. All the anti-$Fc_\epsilon RII$ mAb used have been described: mAb135, mAb176, mAb168, and mAb64 (28); mAb25 (8); MHM6 (24); mAb3-5 (30).

Stable Transfectants of $Fc_\epsilon RII$ cDNA Mutations. Transfection of $Dhfr^-$ Chinese hamster ovary (CHO) DUKX-B1 cells (31) was performed by the calcium phosphate technique as described (32). After 14 days of Dhfr selection, at least 50 resistant colonies were pooled for analysis. Expression of mutant $Fc_\epsilon RII$ on the cytoplasmic membrane was demonstrated by incubation of glutaraldehyde-fixed cells with the $Fc_\epsilon RII$-specific pAb (1:2000 dilution) at 37°C for 1 hr and subsequent staining with a goat anti-rabbit antibody coupled to peroxidase (Bio-Rad). The same procedure was used for staining of cells with mAb (10 μg/ml), except that a goat anti-mouse (Bio-Rad) second antibody was used.

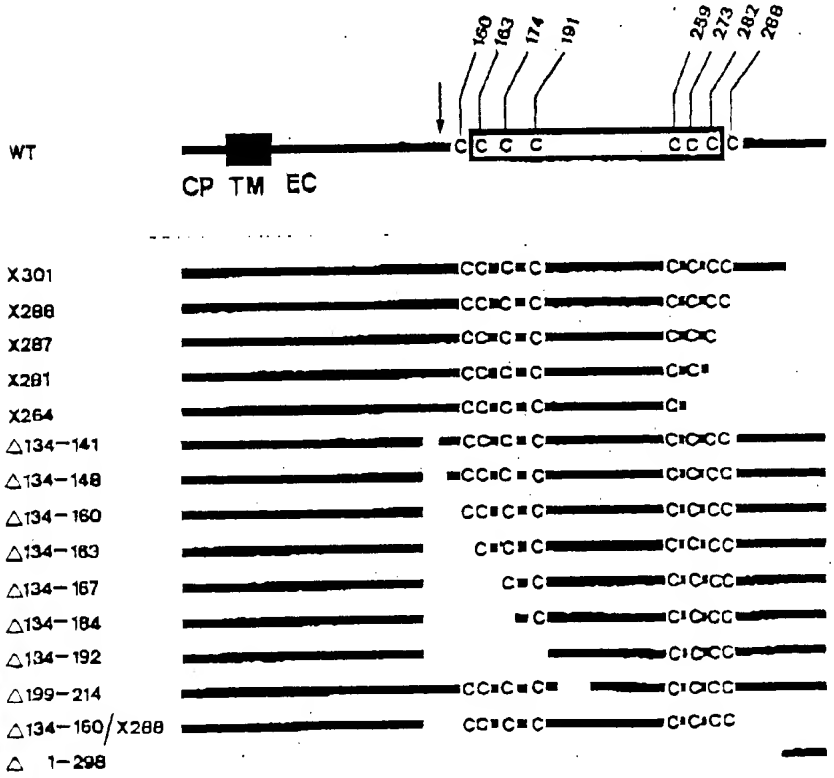

FIG. 2. C-terminal and internal deletions in the $Fc_\epsilon RII$ cDNA. A schematic figure of the entire coding region (wild type, WT) is given at the top. The black box shows the transmembrane (TM) region; the N terminus is exposed to the cytoplasm (CP) and the C terminus is extracellular (EC). The open box represents the cysteine-rich domain homologous with animal lectins. The proteolytic cleavage site for the 25-kDa IgE-BF is indicated with an arrow. Cysteine residues (C) are numbered. The mutants are listed below. Black lines indicate those parts of the protein that are retained in a particular mutant. Deleted sequences are omitted. The name of each mutant is given at the left. Mutants were named as follows. Residues are numbered from the N terminus to the C terminus of the wild-type $Fc_\epsilon RII$. C-terminal truncation mutants (X) contain residues extending from the N terminus through the given amino acid number. Internal deletion mutants (Δ) lack the residues delineated by the numbers. Mutant Δ134–160/X288 encompasses exactly the region containing the 8 extracellular cysteines. A control expression vector, Δ1–298, lacks nearly the entire cDNA insert.

Immunoblot Procedures. Stably transformed CHO cells were harvested and washed in Dulbecco's phosphate-buffered saline without calcium and magnesium. Cellular extracts were obtained after homogenizing the cells in lysis buffer [50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/1 mM $CaCl_2$/1% (vol/vol) Nonidet P-40/0.5% (wt/vol) phenylmethylsulfonyl fluoride] and removing the nuclei by sedimentation at 4000 × *g*. Detergent extracts corresponding to 10^6 cells were subjected to reducing (5% 2-mercaptoethanol in sample buffer) or nonreducing $NaDodSO_4$/12% PAGE (33). The proteins were electrophoretically transferred with a semidry blotter (Sartorius SM17556) to Immobilon membrane (Millipore). The membrane was blocked for 30 min with 50% goat serum in TBST (10 mM Tris·HCl, pH 8.0/150 mM NaCl/0.05% Tween-20/0.1% NaN_3). After overnight incubation with pAb (1:5000) or mAb (mAb135, -176 or -168 at 10 μg/ml; mAb64 at 2 μg/ml; or MHM6 as a 1:500 dilution of ascites fluid) diluted in blocking buffer, the membrane was rinsed in TBST and incubated for 30 min with alkaline phosphatase-labeled goat anti-rabbit or goat anti-mouse antibodies (Bio-Rad), respectively. The membrane was rinsed and developed with the substrates *p*-nitro blue tetrazolium chloride (330 μg/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidine (165 μg/ml) (Bio-Rad) in 100 mM Tris·HCl, pH 9.5/100 mM NaCl/5 mM $MgCl_2$.

Rosetting Assay. A 1:10 dilution of latex beads (Sigma LB-11, diameter 1.09 μm) was incubated with BSA-NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid coupled to bovine serum albumin, 0.3 mg/ml; ref. 34) in 15 mM sodium acetate (pH 5.0) for 1 hr. After two washes in phosphate-buffered saline, the beads were incubated for 1 hr in RPMI 1640 medium (GIBCO) supplemented with 15% fetal bovine serum. The beads were washed twice and resuspended in RPMI 1640 with 15% fetal bovine serum and specific anti-NIP IgE (20 μg/ml). After washing, the IgE-coated beads were sedimented onto stably transformed CHO cells (10^4 cells per well of a 96-well plate) by centrifugation at 200 × *g*. All these steps were done at room temperature. After 2 hr of incubation on ice, unbound beads were removed by gentle washing. The specificity of the rosetting was controlled by inhibition of the reaction with IgE (1 mg/ml) or $Fc_\epsilon RII$-specific pAb (1:5000 dilution).

RESULTS

Design of $Fc_\epsilon RII$ Deletion Mutants. A striking feature of $Fc_\epsilon RII$ is a region of 123 amino acid residues that shows a pronounced homology with the carbohydrate-binding domain of animal C-type lectins. This homology domain is completely contained within the 25-kDa IgE-BF released from the $Fc_\epsilon RII$ by proteolytic cleavage (16–18). The IgE-BF encompasses 8 cysteines, of which 6 are part of the homology domain and the remaining 2 flank it very closely (21, 22). To delimit functional domains, in particular the one for IgE binding, we constructed internal and C-terminal deletion mutants that lack the cysteines in a progressive order (Fig. 2).

Expression of $Fc_\epsilon RII$ Mutants in CHO Cells. Mutant and wild-type $Fc_\epsilon RII$-cDNA expression was driven by the very strong MCMV enhancer/promoter system (Fig. 1). The expression plasmids were used to stably transform $Dhfr^-$ CHO cells by selection for the $Dhfr^+$ phenotype. All the mutated $Fc_\epsilon RII$ were transported to, and correctly inserted into, the cytoplasmic membrane. This was demonstrated by staining the cells with pAb raised against the C-terminal part of $Fc_\epsilon RII$ expressed in *E. coli* (data not shown). The pAb detected wild-type as well as mutant $Fc_\epsilon RII$ but did not stain control cells transformed with plasmid Δ1–298. Expression of mutant $Fc_\epsilon RII$ at the cell surface was estimated by visually comparing the intensity of cell staining with anti-$Fc_\epsilon RII$ antibodies. The intensity of cell surface staining correlates with the expression of mutant $Fc_\epsilon RII$ detected by im... blotting (see below). Staining was generally weake... C-terminal mutants than for internal deletion mutants. P... do not provide a good means to estimate the amount of mutant $Fc_\epsilon RII$ produced by the cells, since removal of epitopes and weak expression cannot be distinguished. All internal deletion mutants were present on the cytoplasmic membrane at about the wild-type level, as estimated by

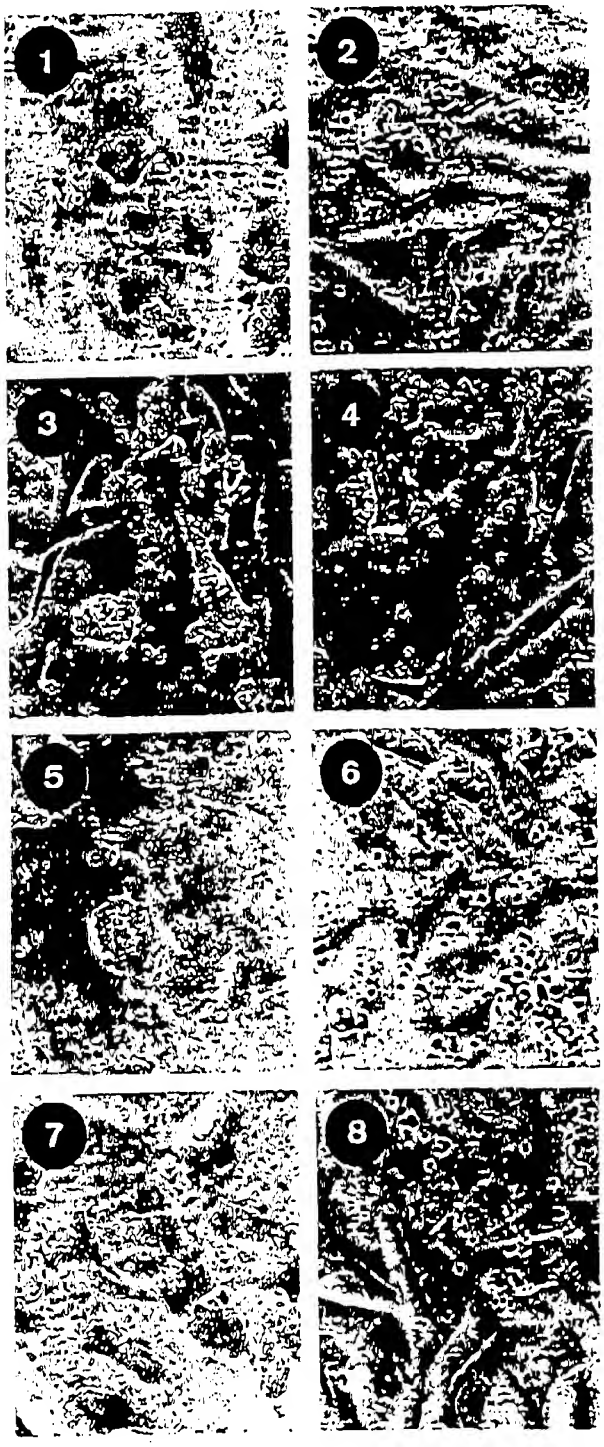

FIG. 3. IgE binding of $Fc_\epsilon RII$ mutants. CHO cells stably transformed with wild-type or mutant $Fc_\epsilon RII$-cDNAs were analyzed by IgE-rosette formation with IgE-coated latex beads. Only those mutants delimiting the breakpoints of IgE-binding activity are shown. (*1*) Wild type. (*2*) Control Δ1–298. (*3*) Mutant X287. (*4*) Mutant X281. (*5*) Mutant Δ134–163. (*6*) Mutant Δ134–160. (*7*) Double mutant Δ134–160/X288. (*8*) Double mutant Δ134–160/X288 preincubated with IgE (1 mg/ml).

staining of the cells with mAb3-5, which recognizes a C-terminal epitope (see below). Unfortunately, there is no mAb available that recognizes all mutant $Fc_\epsilon RII$. Therefore, we cannot exactly determine, in the case of the C-terminal mutants, the relative amount of mutant $Fc_\epsilon RII$ expressed at the cell surface.

Mapping the Region of $Fc_\epsilon RII$ Essential for IgE Binding. Rosetting with immunoglobulin-coated erythrocytes (or beads) is a standard method to demonstrate expression of low-affinity Fc receptors. In our experiments, the specificity of IgE rosettes was checked by inhibition of rosette formation with pAb (data not shown) and IgE (Fig. 3). The results of the rosetting experiments are shown in Fig. 3 and summarized at the top of Fig. 4. They indicate that the C-terminal 34 residues, including Cys-288, can be removed (mutant X287) without an effect on IgE-rosette formation. Further deletion of 6 amino acid residues, which includes removal of a second cysteine at position 282 (mutant X281), impaired IgE binding. Staining of cells with pAb is about equal for these two mutants (data not shown). We therefore conclude that lack of IgE-rosette formation in mutant X281 is not due to a significant reduction of mutant $Fc_\epsilon RII$ expression at the cell surface but represents a real functional inactivation. Internal deletion mutants lost IgE-binding activity when the deletion progressed through Cys-160 (mutant Δ134–163).

It could be argued that the alterations introduced within the IgE-BF region influence binding of IgE by perturbing the overall $Fc_\epsilon RII$ structure. This is unlikely, since it is known that the soluble IgE-BF retains the site for binding of IgE (and also mAb, see below) (16, 18). Hence, our mutational analysis indicates that the $Fc_\epsilon RII$ region between residues 160 and 287 is sufficient for IgE binding.

Epitope Mapping of anti-$Fc_\epsilon RII$ mAb. Extracts prepared from cells expressing wild-type and mutant $Fc_\epsilon RII$ were analyzed on immunoblots with pAb and mAb (Fig. 4). All the mutant proteins were recognized by pAb. As expected, progressive deletion resulted in faster mobility of mutant proteins on $NaDodSO_4$/PAGE under reducing conditions. However, under nonreducing conditions, mutants that had cysteine residues removed behaved anomalously. C-terminal deletion mutants (Fig. 4*A*) shifted to higher molecular weights when Cys-288 was removed (mutant X287). The same effect was seen with internal deletion mutants (Fig. 4*B*) when the first cysteine, Cys-160, was removed (mutant Δ134–163). This behavior on $NaDodSO_4$/PAGE indicates that cysteines 160 and 288 are engaged in disulfide bridges and that their deletion unfolds $Fc_\epsilon RII$.

Epitope mapping of the mAb was done under nonreducing conditions because reduced wild-type and mutant $Fc_\epsilon RII$ proteins are not recognized by the mAb tested. MHM6 is an agonistic mAb delivering to B cells a signal indistinguishable from the effects of low molecular weight B-cell growth factor (BCGF) (5, 11, 12). All other mAb investigated are known to inhibit IgE binding to $Fc_\epsilon RII$ (8, 28); mAb135, -176, -168, and -64 are known to belong to different epitope families (G. Delespesse, personal communication). Additionally, $F(ab')_2$ fragments of mAb135 (9) and mAb25 (10) suppress the IgE synthesis induced by interleukin 4, as well as the ongoing production of IgE by B cells isolated from atopic donors. As shown in Fig. 4, all mAb except mAb3-5 recognize the region delimited by cysteines 163 and 288. In contrast to IgE, the binding of these mAb depends on Cys-288 but not on Cys-160. mAb3-5 is an exception, since it binds the reduced $Fc_\epsilon RII$ and inhibits IgE binding only marginally (30). Its inability to bind mutant X301 indicates that mAb3-5 recognizes the C-terminal 20 amino acid residues of $Fc_\epsilon RII$. Epitope mapping on immunoblots was confirmed by direct staining of cells expressing mutant $Fc_\epsilon RII$ (data not shown).

Expression of a Minidomain That Binds Both IgE and mAb. In summary, our results establish that the amino acid sequence between positions 160 and 288 is critical for binding both IgE and anti-$Fc_\epsilon RII$ mAb (except mAb3-5). We there-

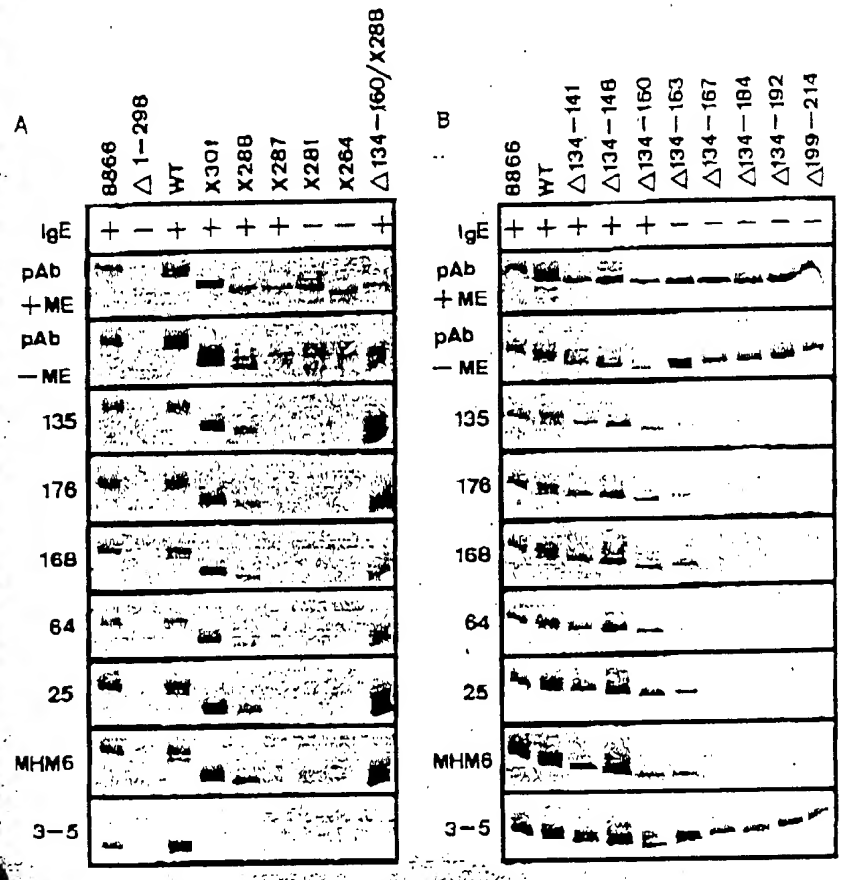

FIG. 4. Epitope mapping of anti-$Fc_\epsilon RII$ mAb by immunoblot analysis. Solubilized cellular extracts were prepared from stably transformed CHO cells producing wild-type (WT) or mutant $Fc_\epsilon RII$. Additionally, $Fc_\epsilon RII$ obtained from the B-lymphoblastoid cell line RPMI 8866 (8866) is shown. The top row of the figure indicates which of the $Fc_\epsilon RII$ mutants do (+) or do not (−) form IgE rosettes as shown in Fig. 3. Immunoblot analysis is shown for pAb, mAb135, mAb176, mAb168, mAb64, mAb25, MHM6, and mAb3-5. For immunoblots with pAb, proteins were separated by reducing (+ME) and nonreducing (−ME) $NaDodSO_4$/12% PAGE. For immunoblots with mAb, electrophoresis was carried out under nonreducing conditions. (*A*) C-terminal deletion mutants. (*B*) Internal deletion mutants.

fore constructed mutant Δ134–160/X288, which encompasses exactly this region. As expected, the domain expressed in this mutant $Fc_\epsilon RII$ is sufficient both for IgE binding (Fig. 3) and for binding all strongly inhibitory mAb and the agonistic mAb MHM6 (Fig. 4*A*).

DISCUSSION

The $Fc_\epsilon RII$, in contrast to other Fc receptors, has not evolved as a member of the immunoglobulin gene superfamily but shows substantial homology with several animal lectins (21, 22). The functional analysis of deletion mutants presented here delimits the IgE-binding domain of $Fc_\epsilon RII$ to a region of 128 amino acids (residues 160–287). Strikingly, the sequence homology between $Fc_\epsilon RII$ (residues 163–282) and the carbohydrate-binding domain of lectins described previously is confined to the IgE-binding domain experimentally defined in this study. The core region of homology, which includes 4 perfectly conserved cysteines, is distributed over a region of 92 amino acids comprising residues 191–282 of $Fc_\epsilon RII$ (21, 22). This region of homology has all the features characteristic of C-type lectins, which are known to bind carbohydrates in a Ca^{2+}-dependent manner and to have conserved intramolecular disulfide bridges (35). In several lectins, such as the asialoglycoprotein receptors, the homology extends 30 residues further toward the N terminus and includes 2 additional conserved cysteines (21, 22). The conservation of the cysteines suggests that $Fc_\epsilon RII$ and lectins have a similar folding of their homology domain. Our results indicate that the correct folding of the $Fc_\epsilon RII$ homology domain is critical for function, since deletion of the cysteines has a deleterious effect on IgE binding.

Recently, Vercelli *et al.* (36) mapped the $Fc_\epsilon RII$ binding site on human IgE to a motif in the $C_{\epsilon 3}$ constant-region domain that is formed on dimerization of one or both of the flanking $C_{\epsilon 2}$ and $C_{\epsilon 4}$ domains. Intriguingly, their results with enzymatically deglycosylated IgE indicated that carbohydrates are not necessary for recognition of IgE by $Fc_\epsilon RII$. It is evident that although IgE is a heavily glycosylated immunoglobulin and the binding domain of $Fc_\epsilon RII$ is the lectin module, a non-lectin-type interaction takes place. Thus, the ancestral lectin module, conceived as a stable structural domain held together by disulfide bridges between conserved cysteine residues, has evolved in $Fc_\epsilon RII$ to recognize the protein moiety of IgE. This unexpected finding is of interest in the light of the recent discovery in the hematopoietic system of a family of adhesion receptors containing a lectin-like domain (24). It is conceivable that lymphocyte migration is not exclusively controlled through carbohydrate-dependent recognition processes as has been proposed, but that the lectin-like domain of these adhesion receptors also displays a non-lectin activity. Conversely, it is also possible that the role of $Fc_\epsilon RII$ in cell adhesion could be the result of a lectin function. It has already been proposed that $Fc_\epsilon RII$ has two binding sites, one for IgE and one for carbohydrates (37). Our result would then indicate that both ligands bind within the same structural domain, raising the possibility of regulation by competitive or allosteric mechanisms.

It has been suggested that $Fc_\epsilon RII$ is the receptor for low molecular weight BCGF (5, 11–13). MHM6, an anti-$Fc_\epsilon RII$ mAb, interferes with BCGF uptake onto cells. This antibody also triggers an effect similar to BCGF, possibly by enhancing the processing of $Fc_\epsilon RII$ into growth-promoting cleavage products (5). Further, it has been demonstrated that the growth-promoting effect of MHM6 is epitope-restricted and that Fab fragments of this mAb are also agonists of BCGF activity. This, together with our present finding that the MHM6 epitope maps to the lectin domain, suggests that this domain is the target for triggering B-cell growth. Therefore, the lectin-like domain may represent a focus for interaction between general B-cell growth and isotype-specific processes, linking together these two so far seemingly unrelated B-cell regulatory pathways and providing a means for regulation at both levels.

We are grateful to H. Rink for providing synthetic oligonucleotides; to Dr. A. Schmitz for expression of IgE-BF in *E. coli*; to Dr. E. Kilchherr for purification of recombinant IgE-BF; to Drs. J. Banchereau, G. Delespesse, J. Gordon, and T. Kishimoto for gifts of mAb; and to Drs. N. Hardman, G. Bilbe, and U. Suter for critically reading the manuscript.

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Exhibit C

(Cite as: 1996 WL 1771384 (Bd.Pat.App & Interf.))

*1 THIS OPINION WAS NOT WRITTEN FOR PUBLICATION

Board of Patent Appeals and Interferences

Patent and Trademark Office (P.T.O.)
EX PARTE PETER A. VANDENBERGH, SHIRLEY A. WALKER AND BLAIR S. KUNKA
Appeal No. 94-2059
Application 07/840,503 [FN1]
December 30, 1996

Ian C. McLeod

2190 Commons Parkway

Okemos, MI 48864

Before WINTERS, GRON and WEIMAR

Administrative Patent Judges.

WINTERS

Administrative Patent Judge.

ON BRIEF

DECISION ON APPEAL

This appeal was taken from the examiner's decision refusing to allow claims 22 and 24-31. Claims 1-21, which are the only other claims remaining in the application, stand withdrawn from further consideration by the examiner as directed to a non-elected invention.

Representative Claim

Claim 22, which is illustrative of the subject matter on appeal, reads as follows:

22. A method for producing a bacteriocin in a growth medium which comprises: (a) culturing live cells of Lactococcus lactis NRRL-B-18535 in a growth medium for the cells so as to produce the bacteriocin in the growth medium, and wherein the bacteriocin contains a protein having a molecular weight of about 6000 daltons, is inactivated by protease V and not inactivated by alpha-chymotrypsin, trypsin, lipase, pepsin and lysozyme, wherein the bacteriocin inhibits the growth of bacteria selected from the group consisting of Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus carnosus, Pediococcus pentosaceus, Pediococcus acidilactici, Lactococcus cremoris, Lactococcus lactis, Leuconostoc mesenteroides, Lactobacillus bulgaricus, Lactobacillus fermentum, Lactobacillus

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bifermentans, Lactobacillus plantarum and Listeria monocytogenes and has a pH for inhibition between about pH 2 and 8; and
(b) separating the bacteriocin in the growth medium from the cells.

The Reference

The prior art reference cited and relied on by the examiner is:
Geis et al. (Geis), "Potential of Lactic Streptococci to Produce Bacteriocin," Applied and Environmental Microbiology, Vol. 45, No. 1, pp. 205-211 (January 1983).

The Issues

In the final rejection, paper No. 6, pages 2 and 3, the examiner rejected claims 22 and 24-31 under 35 U.S.C. 112, first paragraph, as based on a non- enabling disclosure. At pages 3 and 4 of the final rejection, the examiner further rejected claims 22 and 24-31 under 35 U.S.C. 112, first and second paragraphs. Those rejections have been withdrawn. See the advisory action, paper No. 12, and see the answer, page 2, section (4) entitled "Issues."

The issues remaining for review are: (1) whether the examiner erred in rejecting claims 22 [FN2], 24 and 26 under 35 U.S.C. 112, second paragraph, as indefinite; (2) whether the examiner erred in rejecting claims 22 and 24-28 under 35 U.S.C. 102(b) as anticipated by or, in the alternative, under 35 U.S.C. 103 as unpatentable over Geis; and (3) whether the examiner erred in rejecting claims 29-31 [FN3] under 35 U.S.C. 103 as unpatentable over Geis.

Deliberations

*2 Our deliberations in this matter have included evaluation and review of the following materials: (1) the instant specification, including Figure 1, and all of the claims on appeal; (2) appellants' brief before the Board; (3) the examiner's answer; (4) the Geis reference; (5) the Vandenbergh declaration, filed in parent Application Serial No. 07/492,969 under the provisions of 37 CFR 1.132, executed July 16, 1991; and (6) the Vandenbergh "supplemental" declaration, filed in this application under the provisions of 37 CFR 1.132, executed March 8, 1993.

On consideration of the record, including the above-listed materials, we reverse the examiner's rejections under 35 U.S.C. 112, second paragraph, 35 U.S.C. 102(b) and 35 U.S.C. 103.

35 U.S.C. 112, Second Paragraph

According to the examiner, claims 22, 24 and 26 are "essential" duplicates. See the answer, page 3, line 21. Manifestly, however, these claims differ in scope and are not duplicates. Each claim differs from the others with respect to the scope of the subject matter sought to be patented. The claims are not identical. Nor is

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this a situation where the invention is obscured by a large number of claims. Accordingly, a rejection on the ground of multiplicity is improper. See the Manual of Patent Examining Procedure (M.P.E.P.), section 2173.05(n) (6th edition, revision 2, July 1996).

Even assuming arguendo that the claims did not differ in scope, and we hold that they do, nevertheless, the examiner's rejection under 35 U.S.C. 112, second paragraph, would not be sustainable. As stated in Tandon Corp. v. U.S. International Trade Commission, 831 F.2d 1017, 1023, 4 USPQ2d 1283, 1288 (Fed. Cir. 1987) (citation omitted):

> At the same time, practice has been long recognized that "claims may be multiplied...to define the metes and bounds of the invention in a variety of different ways." Thus two claims which read differently can cover the same subject matter.

Furthermore, attention is invited to Hormone Research Foundation Inc. v. Genentech Inc., 904 F.2d 1558, 1567 n. 15, 15 USPQ2d 1039, 1047 n. 15 (Fed. Cir. 1990), where the court stated:

> It is not unusual that separate claims may define the invention using different terminology, especially where (as here) independent claims are involved.

Simply stated, the examiner has not sustained her initial burden of establishing that claims 22, 24, and 26 are prima facie indefinite within the meaning of 35 U.S.C. 112, second paragraph. The rejection of those claims under that statutory provision is reversed.

35 U.S.C. 102(b)/35 U.S.C. 103

In rejecting all of the appealed claims on prior art grounds, the examiner focuses attention on these specific bacteriocin-producing strains disclosed by Geis, namely, S. lactis 5D8, 6F3 and 6F5. See particularly the Geis reference, page 207, Table 2; page 208, Table 3; and page 209, Table 4. According to the examiner, "[t]hese strains are indistinguishable from appellants' claimed strain [L. lactis NRRL-B-188535]" and "the bacteriocin produced by the strains [those of Geis and appellants] appears to be the same." See the answer, page 4, first full paragraph. The examiner further argues that S. lactis 5D8, 6F3 and 6F5 "produce bacteriocins which are not patentably distinct, if not identical, to appellants' bacteriocin" and "the bacteriocins produced by the designated strains ... possess a spectra of inhibitory activity as 'broad' as appellants' claimed bacteriocin." See the answer, paragraph bridging pages 6 and 7. We disagree.

*3 To the extent that the examiner's rejection is predicated on 35 U.S.C. 102(b) (claims 22 and 24-28), we find that (1) the prior art strains 5D8, 6F3 and 6F5 are not identical to appellants' strain L. lactis NRRL-B- 18535, and (2) the bacteriocins produced by strains 5D8, 6F3 and 6F5 are not identical to the bacteriocin recited in appellants' claims. This follows from a comparison of appellants' specification and claims with the Geis disclosure. The claimed method comprises culturing live cells of L. lactis NRRL-B-18535 to produce a bacteriocin, wherein the bacteriocin inhibits the growth of a wide spectrum of bacteria including, inter alia, S. aureus. In contrast, Geis discloses that the bacteriocins produced from S. lactis 5D8, 6F3 and 6F5 possess no activity against S. aureus. See the Geis reference, page 209, Table 4. Thus, the prior art strains produce bacteriocins which do not inhibit the growth of S. aureus whereas

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appellants' strain produces a bacteriocin which inhibits the growth of S. aureus. It follows that the method disclosed by Geis is not identical to the claimed method.

In the answer, page 5, first paragraph, the examiner argues that "[t]he negative reaction with S. aureus [reported by Geis in Table 4] may be due to differences in assay conditions as well as the specific S. aureus strains tested." That assessment, however, is speculation. Where, as here, appellants disclose and claim a method of producing a bacteriocin which inhibits the growth of S. aureus and Geis specifically discloses a bacteriocin showing no activity against S. aureus, the Geis disclosure does not support a finding of prima facie anticipation or shift the burden of persuasion to appellants to rebut any such prima facie case. See the answer, page 5, second paragraph. The rejection of claims 22 and 24-28 under 35 U.S.C. 102(b) as anticipated by Geis is reversed.

Respecting the rejection of claims 22 and 24-31 under 35 U.S.C. 103, we begin with the statute which requires consideration of the claimed subject matter as a whole. As stated in M.P.E.P. section 2116.01 (6th edition, revision 2, July 1996), **all** the **limitations** of a claim must be **considered** when weighing the differences between the claimed invention and the prior art in determining the obviousness of a **process** or **method** claim.

Here, for the reasons previously set forth, the prior art does not establish that strains 5D8, 6F3 and 6F5 are identical to appellants' strain L. lactis NRRL-B-18535. The bacteriocins produced by strains 5D8, 6F3 and 6F5 are not identical to the bacteriocin recited in appellants' claims. Furthermore, the Geis reference provides no suggestion which would have led a person having ordinary skill in the art from "here to there," i.e., from the Geis method for producing bacteriocins to the claimed method for producing another bacteriocin. See Ex parte Tanksley, 37 USPQ2d 1382, 1386 (Bd. Pat. App. & Int. 1994). Nor does the examiner rely on any other prior art reference or references which would cure the above-noted deficiency of Geis. The rejection of claims 22 and 24-31 under 35 U.S.C. 103 as unpatentable over Geis is reversed.

Conclusion

*4 In conclusion, for the reasons expressed in the body of this opinion, we reverse the examiner's rejections under 35 U.S.C. 112, second paragraph, 35 U.S.C. 102(b) and 35 U.S.C. 103. The examiner's decision refusing to allow claims 22 and 24-31, is reversed.

REVERSED

BOARD OF PATENT APPEALS AND INTERFERENCES

SHERMAN D. WINTERS

Administrative Patent Judge

TEDDY S. GRON

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Administrative Patent Judge

ELIZABETH C. WEIMAR

Administrative Patent Judge

FN1. Application for patent filed February 24, 1992. According to appellants, this application is a continuation-in-part of Application 07/492,969 filed March 13, 1990, now abandoned.

FN2. In the answer, page 3, line 17, the reference to canceled claim 23 constitutes an inadvertent error. See the final rejection, page 4, line 13.

FN3. In the answer, page 6, line 3, the reference to canceled claim 32 constitutes another examiner error. See the final rejection, page 5, line 15.

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